MONOGRAPHS ON BIOCHEMISTRY

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GENERAL PREFACE.

THE subject of Physiological Chemistry, or Biochemistry, is enlarging its borders to such an extent at the present time, that no single textbook upon the subject, without being cumbrous, can adequately deal with it as a whole, so as to give both a general and a detailed account of its present position. It is, moreover, difficult in the case of the larger textbooks to keep abreast of so rapidly growing a science by means of new editions, and such volumes are therefore issued when much of their contents has become obsolete.

For this reason an attempt is being made to place this branch of science in a more accessible position by issuing a series of monographs upon the various chapters of the subject, each independent of and yet dependent upon the others, so that from time to time, as new material and the demand therefor necessitate, a new edition of each monograph can be issued without re-issuing the whole series. In this way, both the expenses of publication and the expense to the purchaser will be diminished, and by a moderate outlay it will be possible to obtain a full account of any particular subject as nearly current as possible.

The editors of these monographs have kept two objects in view: firstly, that each author should be himself working at the subject with which he deals; and, secondly, that a *Bibliography*, as complete as possible, should be included, in order to avoid cross references, which are apt to be wrongly cited, and in order that each monograph may yield full and independent information of the work which has been done upon the subject.

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THE PROTAMINES AND HISTONES

BY THE LATE

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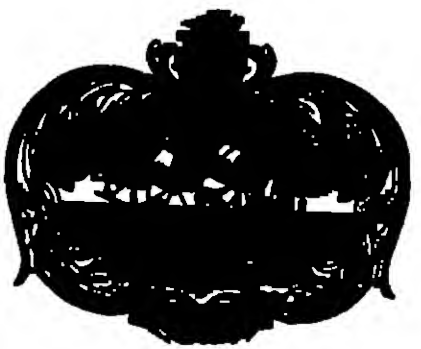

LONGMANS, GREEN AND CO.
LONDON ◆ NEW YORK ◆ TORONTO
1928

Made in Great Britain

PREFACE.

My father devoted the greater part of his work during the last months of his life to this monograph. The invitation of the editors of *Monographs on Biochemistry* coincided with his wish to set forth comprehensively his own particular sphere of work, and it was granted to him just to finish the work in which he was engrossed. A few days before his death he told me that the manuscript was ready for the press. The text is thus all his own work. The "Contents" was completed according to his design from the final text. Only the Preface remained unfinished. However, a rough draft of it was found, which, in spite of its evidently incomplete form, shows those points which should be emphasised in the preface. It reads :—

"Investigations on protamines and histones have been undertaken chiefly from biological aspects. The author was first led to study the evolutionary changes which protein, one of the chief constituents of the cell, undergoes in the differentiation of the tissues. The change was discovered to consist in the production of proteins which are distinguished by basic properties from the general widely distributed typical proteins, which possess acidic character. The protamines and histones form this class of proteins. They are biologically the more important because they are formed in the chief organ of the cell, the nucleus. They do not, however, occur in all nuclei, but only in the nuclei of certain kinds of tissues. A new and as yet unexplained characteristic was then revealed. Analysis showed that these nucleo-proteins existed in a great variety of chemical structures in the various families and genera of animals, and the question arose whether these chemical forms of karyoplasm were of equal importance to the morphological distinctions in the systematic and evolutionary consideration of the animal kingdom.

"New chemical methods had to be developed for the in-

vestigation of these proteins, and they are described in the first part of this monograph. But, apart from this, the substances found in the karyoplasm are of purely chemical interest. They appear to be, as it were, a protein molecule which is extended in one direction and stunted in another. The extended part, which contains only certain parts of the protein molecule, predominates, and so the whole appears simplified.

"As simple analogues of typical proteins, the protamines are particularly favourable substances for the study of certain properties and structural relations of the proteins.

"Knowledge of the protamines and histones has so far only been derived from the descriptive side, and belongs to the large and important region of biochemistry, which has thus far been advanced and developed only by descriptive and not by experimental means.

"Many of the data go back to the years when there was no clear standpoint for a criticism of the results, and particularly the analytical methods were not developed. Therefore re-examination is desirable."

All to whom I turned to do the last steps which were necessary for publication have helped in the most friendly way. Professor Felix, at the wish of my father, looked through the pages relating to his own work and suggested several small changes in figures, as well as the addition of a sentence. Miss Luise Gruber completed the references in the way intended by my father. Professor Plimmer has undertaken the care of the English edition in the most friendly manner, Dr. Thorpe undertook the translation, and Professor Edlbacher and Professor Felix a revision of the proof-sheets. To them all I wish to express here my most sincere thanks.

W. KOSSEL.

KIEL,
October, 1927.

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PART I.

THE PROTAMINES.

CHAPTER I.

THE MEANING OF THE TERM "PROTAMINE."

The discovery of the protamines was a result of investigations into the chemical nature of the cell nucleus which Friedrich Miescher (126) began in Hoppe-Seyler's laboratory in the year 1868. Miescher found proteins of acidic character containing phosphoric acid—the "nucleins"—in the nuclei of pus cells. He extended his researches to other organs rich in nuclear material, and found in the sperm of the salmon the salt-like compound of a nuclein (later called "nucleic acid") with a base to which he gave the name "protamine" (127). He described its properties and deduced the formula $C_{9}H_{20}N_{5}O_{2}(OH)$ from analysis of the platinum salt. The analyses, repeated and supplemented in the same year by Piccard (142), agree to a certain extent with those of later workers. The simple formula advanced by Miescher could not, however, be maintained.

The results of Miescher attracted little attention because the chief point of interest in these investigations, that is, the analogy between this protamine and the proteins, was overlooked. During the first twenty years after the discovery of protamine only one brief reference to it occurs in the literature (5), and only incidental mention in the text-books.

In 1884 there appeared a paper by Kossel (74) on a protein rich in nitrogen which had been found in the nuclei of the red blood corpuscles of the bird, in salt-like combination with nucleic acid. In this respect it presented an analogy to the protamine of salmon sperm. Kossel called his substance "histone." Further work on the constituents of the nucleus brought out its analogy to protamine more clearly.

Histone occurs in salt-like combination with nucleic acids in other organs rich in nuclear material, such as the thymus gland (Lilienfeld,

1892-94) (118, 119). The final proof of the close relationship between these two substances and the protein-like nature of protamine was given by researches on the hydrolysis products of a base obtained from the sperm of the sturgeon. Kossel (75A, 76) found in this material a substance which was similar to the salmon protamine, but not identical with it. Hence he proposed that the term protamine, which had been introduced by Miescher for the base prepared from salmon sperm, should be used as a general name for both bases, and individual protamines should be named after the family name of the fish—thus salmine and sturine. This nomenclature has been applied to the protamines discovered later, e.g. clupeine and scombrine.

The hydrolysis of sturine carried out by Kossel in 1898 (77, 79) revealed the presence of three basic decomposition products: arginine, lysine, and the then unknown base histidine. Arginine had previously been discovered by Schulze and Steiger (158) in the cotyledons of etiolated lupin seedlings and recognised as a decomposition product of proteins by Hedin (60). Lysine had been found by Drechsel in 1898 (23) also as a unit of the protein molecule. About the same time Hedin (61) obtained histidine by the hydrolysis of other proteins.

The further work of Kossel and his collaborators developed mainly in two directions. The investigations were first extended to the sperm of other fish, and in this way new types of protamines were found.

The methods of separating and isolating the decomposition products were next elaborated. New decomposition products were characterised and the quantitative relations of the units were made clearer.

Kurajeff in 1898 (110) working in Kossel's laboratory found in the testicles of the mackerel a compound which was similar to salmine, but not identical with it. Morkowin (131) prepared from the testicles of Cyclopterus lumpus another protamine of a different type. Others were found in the sperm of the carp, the perch, and Crenilabrus Pavo. It became clear that the occurrence and distribution of the protamines were confined to the testicles and sperm of fish, and that in certain families of fish the protamines were replaced by histones.

Increasing knowledge of the hydrolysis products of the protamines confirmed Kossel's view, which had at first met with some opposition, that the protamines belonged to the protein group and were the most elementary type of this large and important class of compounds. In addition to the above-mentioned bases, arginine, histidine, and lysine, the following monoamino-acids were gradually found among the hydrolysis products: valine, proline, and in certain prota-

mines tyrosine, serine, alanine, and tryptophan. At the present time no decomposition product of protamines is known which does not occur in typical proteins. Also the mode of linkage is the same as exemplified especially by their behaviour with proteolytic enzymes (77). Finally, it has been shown by the study of the origin of protamines in the organism of the salmon family, that typical proteins are converted into protamines in the course of spermatogenesis (128, 196).

The chemistry of the protamines is therefore a part of the chemistry of the proteins. The protamines are the simplest of a series of compounds, the most complex of which are the typical proteins. There are about twenty units in the common proteins but only four in the protamines. The study of the protamines would thus be expected to give important information about the whole group of the proteins. The technique of the quantitative hydrolysis of the protamine molecule into its basic constituents has already been applied to the analysis of complex proteins.

Histones, like the protamines, are a class of compounds comprising various types. They contain a greater number of units and thus are more nearly related to the complex proteins. Their close relationship with the protamines can be demonstrated by histological and chemical means. The process of development, which leads to the formation of protamines in the nuclei of certain families and genera of fish, stops in most animals at an intermediate stage, at the formation of histones. During development changes occur in the original protein which cause it to become basic in character. The histones are the first and the protamines the final stage of these degradative changes. Histones, unlike the protamines, are not confined to one small section of the animal kingdom. Neither protamines nor histones have been found in plants.

CHAPTER II.

THE UNITS OF THE PROTAMINES.

It is well known that the proteins break down on hydrolysis into a large number of compounds which are regarded as the units of the protein molecule. The protamines break down in a similar way on heating with acids or alkalies or by the action of enzymes. Larger fragments of the molecule, composed of two or more units combined together —protones, peptides, or anhydrides—are obtained by less drastic hydrolysis of the protamines.

The following compounds have been identified as units of the protamine molecule :—

(1) Alanine, (2) serine, (3) an aminovaleric acid, (4) proline, (5) an aminocaproic acid, (6) tyrosine, (7) tryptophan, (8) histidine, (9) lysine, (10) arginine.

The methods of investigation are essentially the same as those which are employed for the hydrolytic cleavage of the proteins, but by reason of the smaller number of units the process is considerably simplified. The fact that the action of strong acids produces no humin or ammonia further facilitates the process.

The units of the protamines are partly basic in character and partly neutral in the form of monobasic monoamino-acids ; the analytical procedure will consequently be described in two parts, one dealing with the isolation of the three basic constituents and the other with the detection and estimation of the monoamino-acids.

A. SEPARATION AND QUANTITATIVE ESTIMATION OF THE BASES.

There are a number of methods for the examination of the bases, but the silver-baryta method devised by Kossel and Kutscher in 1900, and its most important modifications, will be described first. It depends on the formation of insoluble silver compounds of arginine and histidine in the presence of fixed alkalies. These compounds had been previously described and used for analysis by Kossel in 1898. Since a protamine seldom contains all three bases, generally only a

part of the analytical method described below is necessary, but in the case of the histones the whole process must be used. The silver-baryta method is also the basis of the methods for the preparation of the individual decomposition products of the protamines. Most of the analyses given in this monograph have been performed by this method. It is therefore given first in its older form and then with Kossel and Gross's method of precipitating arginine with flavianic acid.

(*a*) Silver-Baryta Method.

The method can be divided into the following parts :—

(1) Hydrolysis of the protamines with sulphuric acid.
(2) Removal of sulphuric acid.
(3) Precipitation of arginine and histidine.
(4) Separation of histidine and arginine. Estimation of histidine.
(5) Estimation of arginine.
(6) Estimation of lysine.

(1) *Hydrolysis of the Protamines.*

The following reagents have been employed for hydrolysis :—

(*a*) Boiling sulphuric acid, up to 33 per cent. by volume, concentrated hydrochloric acid, or hydriodic acid [1] at atmospheric pressure.

(*b*) Dilute sulphuric acid at a pressure of from one to four atmospheres and a temperature of 160°.

The conditions required for complete hydrolysis into the decomposition products mentioned above have not yet been sharply defined.

Nelson-Gerhard (133) dissolved salmine sulphate in dilute sulphuric acid so that the solution contained 10 per cent. of protamine sulphate and 6 per cent. (by weight) of sulphuric acid and autoclaved it for two hours at 141° and at a pressure of one atmosphere. The hydrolysed solution gave no biuret reaction, but still contained peptides of monoamino-acids. Higher temperatures (166° to 169°) decomposed the arginine.

Gross (54) found that the hydrolysis of clupeine was incomplete after heating 80 minutes at 160° with sulphuric acid (4 per cent. by volume). The product contained arginine as well as monoamino-acids in peptide combination. The biuret reaction was no longer given. A measure of the hydrolysis was obtained by determination of the free amino-nitrogen.

[1] Iodine and red phosphorous can be used instead of hydriodic acid (see *Zeit. Physiol. Chem.*, 31, 178).

In most cases hydrolysis with 25 to 50 per cent. sulphuric acid (by weight) for 20 hours has been employed. 0·5 to 5 grams of the protamine sulphate are boiled with a mixture of two to three times the weight of concentrated sulphuric acid and four to six times the weight of water for 20 hours under a reflux condenser on a paraffin bath. The liquid is then made up to a known volume and an aliquot part used for the determination of nitrogen by Kjeldahl's method. The micro method is used if only small quantities of material are available. This determination gives the total nitrogen in the substance under investigation. (Nitrogen A.)

(2) *Removal of Sulphuric Acid.*

The solution is warmed and a hot solution of barium hydroxide is gradually added until the liquid is only feebly acid. The barium sulphate precipitate is filtered off, boiled up with water four times and washed until the washings no longer give a precipitate with phosphotungstic acid. The combined filtrate and washings are evaporated and made up to a known volume. The nitrogen adhering to the barium sulphate is calculated by estimating the nitrogen (by Kjeldahl's method) in an aliquot part of the solution. (Nitrogen B.)

(3) *Precipitation of Arginine and Histidine as Silver Compounds.*

The solution is placed in a flask and gradually treated with a boiling solution of silver sulphate with constant stirring. The quantity of reagent required is ascertained in the following way: A drop of the liquid is transferred by means of a glass rod to a drop of baryta on a watch glass, standing on a black surface. If a white or yellow precipitate is formed, more silver sulphate is required. If the precipitate is brown, sufficient reagent has been added. When this is the case, powdered baryta is added to the cooled liquid until some remains undissolved on the bottom of the vessel even after long stirring. The voluminous brown precipitate is filtered off and ground up with the filter paper in a large mortar with sand and baryta solution, refiltered and thoroughly washed with baryta solution. The precipitate contains the arginine and histidine; lysine and monoamino-acids are present in the filtrate.

(4) *Separation of Histidine and Arginine.*

The precipitate from (3) is suspended in sufficient dilute sulphuric acid to give a feebly acid solution and decomposed with hydrogen

sulphide. The liquid is boiled to remove hydrogen sulphide and the precipitate of silver sulphide and barium sulphate filtered off, boiled with water and washed until there is no precipitate with phosphotungstic acid. The combined filtrate and washings are evaporated and made up to a known volume. From a Kjeldahl estimation in an aliquot portion the nitrogen precipitated by silver and baryta can be calculated. (Nitrogen C.) Another aliquot portion is used for carrying out the diazo reaction. If this is positive, histidine is present and the procedure in the next paragraph is adopted. If negative, the estimation is carried out according to Section (5).

The separation of histidine and arginine depends upon the fact that the silver compound of histidine is precipitated at a less alkaline reaction than that of arginine. If a neutral solution of a mixture of the silver compounds of histidine and arginine is gradually made alkaline by the addition of baryta, a point is reached at which the precipitation of histidine is complete, while the arginine has not yet started to separate. This is achieved by the very cautious addition of baryta.[1] An easy way of reaching this end point is to add barium carbonate to the neutral solution of the silver compounds of the bases and heating. Then the alkalinity [2] produced by the arginine carbonate is sufficient to cause the precipitation of the silver compound of histidine without any separation of the arginine. The complete precipitation is thus in this case dependent on the presence of arginine.

The procedure is carried out in the following way: The filtrate from the silver sulphide is neutralised with baryta and treated with barium nitrate to complete the precipitation of the sulphuric acid. The barium sulphate is filtered off, washed, and the solution evaporated to about 100 c.c. After acidifying with nitric acid, concentrated silver nitrate is gradually added until a test drop gives a brown precipitate with baryta. Baryta solution is now added until the solution is feebly acid, and then a suspension of barium carbonate. The solution is heated on the water bath and then just brought to the boil over a gauze. The precipitate contains all the histidine and, after cooling, is filtered off and washed with weak baryta solution until free from nitric acid. The filtrate and washings are kept for the estimation of arginine. The precipitate is acidified with sulphuric acid,

[1] "After the separation of the silver compound of histidine is ended, the gradual further addition of baryta at first produces no precipitation, but a large excess of baryta precipitates the silver compound of arginine" (Kossel and Kutscher, 1900), (80).

[2] Vickery and Leavenworth (1918) give pH 7·0 as the approximate hydrogen ion concentration and describe a method of attaining this by the use of bromthymol blue.

gently warmed and decomposed with hydrogen sulphide. The filtrate and washings from the silver sulphide are reduced to 100 c.c. The nitrogen in an aliquot portion is estimated by Kjeldahl's method. (Nitrogen D.) From this the amount of histidine can be calculated, making allowance for the various portions removed for analyses.

Gravimetric Estimation of Histidine.

The gravimetric estimation of the histidine is made with the rest of the liquid which is freed from sulphuric acid by hot baryta solution, the excess of baryta being removed with carbon dioxide. The barium sulphate and carbonate are filtered off and well washed, and the filtrate and washings evaporated to about 10 c.c. The histidine is converted into the monopicrolonate (71) (see also Brigi, 14) by adding slightly more than one molecule of picrolonic acid, dissolved in alcohol, i.e. an amount corresponding to one-third of the nitrogen found in D. The picrolonate is filtered off after three days, washed with a little water and dried at 100°. The histidine in the picrolonate is calculated from the formula $C_6H_9N_3O_2 . C_{10}H_8N_4O_5$. Vickery and Leavenworth (1928) recommend using histidine flavianate for this purpose.

(5) *Estimation of Arginine.*

This estimation is performed on the filtrate from the silver compound of histidine (above).

If histidine is absent, the nitrogen C gives the arginine nitrogen.

If histidine is present, a further precipitation of the arginine as silver salt is necessary. The filtrate from the histidine silver compound is saturated with powdered baryta. The precipitate is filtered off and, with the filter paper, ground up with baryta and sand, and washed until free from nitric acid. The precipitate is then suspended in water, acidified with sulphuric acid and decomposed with hydrogen sulphide. The filtrate and washings from the silver sulphide are evaporated to a known volume and the nitrogen estimated in an aliquot part. (Nitrogen E.) The amount of arginine is calculated from this estimation.

Gravimetric Estimation of Arginine.

The liquid, in which the nitrogen C (in absence of histidine) or E (if histidine is present) was determined, is used for the gravimetric estimation of arginine. This estimation consists in the conversion of the arginine into the sparingly soluble "flavianate," the salt of 1-naphthol-2, 4 dinitro-7 sulphonic acid (104).

The greater part of the sulphuric acid is removed from the solution by adding baryta until the reaction is only just acid to Congo paper. The presence of barium salts in the solution must be avoided, but a slight excess of sulphuric acid does not interfere. The precipitate of barium sulphate is filtered off and extracted four times with hot water. The combined filtrate and washings are reduced to a known volume. A portion of this solution containing from 0·02 to 0·05 gram nitrogen (0·06 to 0·15 gram arginine) is treated with aqueous flavianic acid, 15 parts by weight being required for every part of nitrogen (calculated from C or E). The liquid is made up to 50 c.c. and left to stand for three days. The arginine flavianate is then filtered off in a weighed Gooch crucible and washed with water containing a trace of flavianic acid. A very small amount of arginine flavianate goes into solution, increasing the yellow colour of the wash water. The washing is complete when successive portions of the washings match in colour. The arginine flavianate is dried at 105°. One part by weight corresponds to 0·3566 parts of arginine.

(6) *Estimation of Lysine.*

The filtrate from the first silver precipitate obtained in (3) is acidified with sulphuric acid to remove barium and freed from silver with hydrogen sulphide. The precipitate of barium sulphate and silver sulphide is filtered off and washed. The combined filtrate and washings are reduced to a volume of from 50 to 200 c.c. according to the quantity of the substance under examination. Two aliquot parts of this solution are taken. In one the nitrogen is estimated by Kjeldahl's method (nitrogen F). The other is acidified with sulphuric acid and treated with a drop of phosphotungstic acid solution; if a precipitate comes down at once or after a short time lysine is present. If no precipitate is formed, lysine is absent and F gives the nitrogen of the monoamino-acids. If lysine is present, the whole liquid is treated with sulphuric acid so as to contain about 4 per cent. sulphuric acid and phosphotungstic acid is added, a large excess being avoided. The reagent is added until a test drop of the clear liquid remains clear 10 seconds after the addition of more phosphotungstic acid. After 24 hours the precipitate is filtered off, ground up in a mortar and thoroughly washed with 4 per cent. sulphuric acid. The filtrate and washings are reduced to a known volume and the nitrogen in an aliquot part is estimated by Kjeldahl's method. This gives the nitrogen not precipitated by phosphotungstic acid. (Nitrogen G.)

Gravimetric Estimation of Lysine.

Lysine is converted into lysine picrate for this estimation.

The phosphotungstate precipitate is ground to a uniform paste with water and poured into boiling water. A hot concentrated solution of baryta is then added until the liquid is strongly alkaline. The insoluble barium salt is filtered off, extracted several times with hot baryta and washed with hot water until there is no longer a precipitate with phosphotungstic acid. The filtrate and washings, after passing carbon dioxide to remove excess of barium, are concentrated over a flame, filtered and evaporated to dryness. The residue is taken up in water, filtered from barium carbonate and again evaporated. After addition of alcohol the thick syrupy residue is stirred up with a small quantity of alcoholic picric acid. A concentrated solution of picric acid is then added gradually, at first in very small quantities, to the alcoholic liquid in a porcelain basin until neutral to litmus. The picrate, which separates, is collected after 24 hours and washed with a very little absolute alcohol, dissolved in boiling water, filtered if necessary, and the solution evaporated to a small volume. On cooling, lysine picrate separates in needles which are collected on a weighed Gooch crucible, washed with a little alcohol, dried and weighed.

The combined mother liquors, after removal of alcohol by evaporation, are acidified with sulphuric acid to about 4 per cent. by volume and the picric acid is removed by extraction with ether. After removal of the ether, the aqueous solution is again precipitated with phosphotungstic acid and the precipitate worked up for lysine as above. The process is repeated as long as a precipitate of lysine picrate is obtained with alcoholic picric acid. By careful addition of picric acid in the above precipitations and by avoiding an excess, which redissolves the precipitate, the yield of lysine picrate after the third phosphotungstic acid precipitation is usually so small that it can be neglected. The lysine is calculated from the picrate which has the formula $C_6H_{14}N_2O_2 . C_6H_3N_3O_7$.

The meaning of the nitrogen values A to G is as follows:—

A. Total nitrogen of the protamine.

B. Nitrogen in the filtrate from the first barium sulphate precipitate.

A-B. Nitrogen adsorbed by the barium sulphate precipitate.

C. Nitrogen in the fraction precipitated by silver and baryta (arginine + histidine).

D. Histidine nitrogen.
E. Arginine nitrogen.
F. Nitrogen of lysine + monoamino-acids.
B-(C + F). Nitrogen adsorbed by silver sulphide.
G. Monoamino-acid nitrogen.
F-G. Lysine nitrogen.

The various portions removed for analyses must be taken into account in these calculations.

(7) *Adsorption of Nitrogen Compounds on the Precipitates.*

The adsorbed substances are not washed out by repeated extraction with hot water. The amount of adsorbed nitrogen (A-B) corresponds to 1 to 5 per cent. in the protamines of the salmine group, and a much greater proportion in the other protamines and histones. In the calculation of the results of the analyses the question arises whether the nitrogen of the bases is concerned in this adsorption. If it is not, the ratio of the total nitrogen to the nitrogen of the bases is given by the fraction $\frac{A}{\text{Nitrogen of the Bases}}$, whereas, if all the hydrolysis products are adsorbed equally, the ratio is represented by the fraction $\frac{B}{\text{Nitrogen of the Bases}}$.

In answer to this question Schenck performed the following experiment. In the mixture of the hydrolysis products of cyprinine the ratio of the total nitrogen to the nitrogen of the substances precipitated by phosphotungstic acid was determined without removal of the sulphuric acid required for the hydrolysis, that is, the ratio $\frac{A}{\text{Nitrogen of the Bases}}$. In another part the sulphuric acid was removed and the ratio $\frac{B}{\text{Nitrogen of the Bases}}$ estimated on the filtrate (and washings). Although 4·14 per cent. of nitrogen was adsorbed on the precipitate and could not be washed off, yet the ratio of the total nitrogen to the nitrogen precipitated by phosphotungstic acid showed no change. The adsorption thus concerned equally the basic and non-basic hydrolysis products, and in this case there was no basis for the assumption that one group of substances accounted for all of the adsorbed nitrogen (humin).

Analysis by Staudt show how this adsorption affects the protamines rich in arginine.

up to 100 c.c. Total nitrogen is estimated in 1 c.c. according to Pregl's method. It is heated for 3 hours with sulphuric acid, potassium sulphate, a trace of copper sulphate and a small globule of mercury; 0·01 N solutions are used in the titration.

With protamines of the salmine group, the acid is removed by the addition of baryta until the solution only just blues congo paper. The barium sulphate is filtered off and extracted four times with about 250 c.c. of water. Filtrate and washings are concentrated to 100 c.c.; 1 c.c. of this solution is used for a micro-Kjeldahl estimation. The arginine is estimated by treating a volume of this solution, which is calculated to contain about 0·1 gram of arginine, with a solution of 0·5 gram of flavianic acid, the total volume being made up to 30 c.c.

Lysine is estimated in the filtrate from the silver-baryta precipitate.

(c) VAN SLYKE'S METHOD.

The method of van Slyke has often been used for the analysis of the higher proteins, but has not yet been widely applied to the protamines. The principle of the method is as follows:—

(1) The three hexone bases in the hydrolysate of the protein are precipitated with phosphotungstic acid.

(2) If a mixture of the bases is boiled with strong caustic soda, arginine gives off half of its nitrogen as ammonia, but histidine and lysine form no ammonia under these conditions. Thus the arginine can be calculated from the amount of ammonia formed.

(3) If a mixture of the three bases is treated with acetic acid and potassium nitrite, the α-amino-nitrogen of all three bases and the ε-amino-nitrogen of lysine react according to the following scheme:—

$$RNH_2 + HNO_2 \rightarrow ROH + H_2O + N_2.$$

The nitrogen of the guanidine group of arginine and of the iminazole group of histidine do not take part in the reaction—"non-amino-nitrogen." The difference between the total nitrogen of the bases and the reactive amino-nitrogen gives the value of the non-amino-nitrogen. If the total nitrogen of the arginine is A and the non-amino-nitrogen B, then the histidine nitrogen is $\frac{3}{2}\left(B - \frac{3}{4}A\right)$. The lysine nitrogen is the difference between the total nitrogen of the mixture of the bases and the sum of the nitrogen of the arginine and histidine.

Pilmmer's modification of van Slyke's method is most convenient.[1]

1 to 5 grams [2] of protamine are boiled for 24 hours with ten to twenty times the weight of 20 per cent. hydrochloric acid. The solution is then concentrated *in vacuo* to remove as much hydrochloric acid as possible, dissolved in warm water and made up to a known volume. An aliquot part is used for the estimation of the total nitrogen. 15 c.c. of concentrated hydrochloric acid are added for every 100 c.c. of solution and phosphotungstic acid (prepared according to Wu) in aqueous solution is added until no further precipitate is formed. The liquid is then made up to about 200 c.c. (according to the amount of the phosphotungstate precipitate) and heated until most of the precipitate has dissolved. After standing at room temperature for two days the precipitate is filtered off on a Jena glass filter and washed with 50 to 100 c.c. of dilute hydrochloric acid (1 : 10), using it in portions of 10 c.c., the precipitate being sucked dry after each washing. The filtrate and washings are again filtered through a 7 cm. paper.

The phosphotungstate precipitate is dissolved on the filter in just sufficient N sodium hydroxide and the solution and washings again filtered through the paper used above and made up to 100 c.c.

This solution of the bases is used for :—

(1) The estimation of the ammonia given off by arginine on heating.

(2) The estimation of the total nitrogen of the bases.

(3) The estimation of the amino-nitrogen.

Estimation of the Arginine Nitrogen.—An aliquot part is taken, the actual volume being determined by the arginine content of the solution, and an equal volume of 40 per cent. sodium hydroxide added. This solution is boiled gently for 6 hours under a reflux condenser, using Folin's bulb apparatus for the collection of the ammonia. The water is then run out of the condenser and the liquid boiled for 20 or 30 minutes when the ammonia distils over into the bulbs.

The amount of ammonia formed is estimated by titration. 1 c.c. of 0·1 N acid corresponds to 2·8 mg. of arginine nitrogen.

Estimation of the Total Nitrogen of the Bases.—This is determined on an aliquot part of the solution by the usual Kjeldahl method.

Estimation of the Amino-nitrogen of the Bases.—In another aliquot part of the solution of the bases the amino-nitrogen is estimated in

[1] See also Pilmmer, *Chemical Constitution of the Proteins*, 1, 3rd edition (1917), p. 102; and *Biochem. J.*, 19, 1004 (1925); and Kossler, *J.B.C.*, 42, 267, 1920.

[2] Since the arginine content of the various protamines is very different, no general rule can be given for the amount required for analysis.

van Slyke's micro apparatus. On account of the presence of lysine 1 hour is necessary according to Plimmer at temperatures below 20°.

If the filtrate from the phosphotungstate precipitate is heated with alkali in the above manner, it is found (Plimmer) that some ammonia is formed of which the origin is not yet clear. If this is ascribed to arginine, one must assume that under certain circumstances one-third of the total arginine may escape precipitation by phosphotungstic acid. Plimmer adds the proviso "assuming that no other amino-acid behaving like arginine is present in proteins."

The Nitrogen of the Monoamino-acid Fraction can be obtained from the difference between the total nitrogen and the nitrogen of the bases precipitated by phosphotungstic acid. It can also be obtained directly by a Kjeldahl estimation on the filtrate from the phosphotungstate precipitate.

If the amino-nitrogen is estimated in this filtrate, the non-amino-nitrogen is given by the difference of the total monoamino-acid nitrogen and the amino-nitrogen. The latter is in most of the protamines proline nitrogen (or if hydroxyproline is also present, the nitrogen of proline + hydroxyproline).

B. SEPARATION OF THE MONOAMINO-ACIDS.

The method described above gives a fraction containing the monoamino-acids, of which the nitrogen content is given by the value G (p. 9). Our knowledge of this fraction is not so far advanced as that of the basic hydrolysis products. This is chiefly because there is usually only a small amount of material available for investigation, the weight of monoamino-acids in the more accessible protamines only amounting to one-quarter of that of the bases. Also, the separation of the monoamino-acids is not so sharp as that of the bases.

Kossel and Dakin (82, 83) used the following method for a preliminary separation :—

(1) Extraction of the monoamino-acid fraction with absolute ethyl alcohol : proline is soluble.

(2) Extraction of the residue with methyl alcohol : (*a*) aminocaproic acid, valine and alanine are soluble, (*b*) tyrosine and serine are insoluble.

Fraction 1 contains besides proline small amounts of other substances. These are separated by removing the alcohol and again extracting the dried residue with absolute alcohol. The operation is repeated several times until the residue is completely soluble in alcohol. The alcohol insoluble portions are combined with fraction (2). The alcoholic solution on evaporation gives crystals of *l*-proline, which

is characterised by its melting-point, conversion into the hydantoin (Daikin, 20) or phenylisocyanate compound (E. Fischer, 38) and elementary analysis.

The *l*-proline originally present is partly racemised during the hydrolysis. *l*-Proline forms an alcohol-soluble copper salt which can be separated from the copper salt of *d, l*-proline which is insoluble in alcohol (99). Proline can also be purified by means of its well-crystallised mercuric chloride compound (83).

A nitrogen estimation on the alcohol-soluble fraction gives the proline nitrogen.[1]

Fraction 2.—Tyrosine is recognised by Millon's reaction and its behaviour with diazobenzene sulphonic acid (Pauly's reaction). It separates out from the mixture in characteristic needles.

Often only one of the remaining amino-acids of fractions 2*a* and 2*b* is present in the protamine. In such cases the substance is purified by recrystallisation and identified by elementary analysis. This method can sometimes be employed for a mixture of two amino-acids (83).

If there is a large amount of material, Emil Fischer's esterification method may be used. Melting-points, rotatory power and the phenyl-isocyanate compounds may be used for characterisation.

Up to the present tryptophan has only been detected in protamines by the colour reaction (Hopkins and Cole). Since it is destroyed by acid hydrolysis, it can only be obtained by using trypsin as the hydrolytic agent.

[1] Proline (in absence of hydroxyproline and tryptophan) can also be estimated as the "non-amino-nitrogen" of the monoamino-acid fraction (p. 16).

CHAPTER III.

PREPARATION OF PROTAMINES.

Sperm is the most convenient material for the preparation of protamines, but as this is rarely available in sufficient quantity the *ripe* testicles [1] are generally used. For chemical examination this material has the advantage over most animal organs that a suspension of histologically uniform cells can be readily obtained from it. If unripe testicles are used, this advantage is lost and the results are liable to be very misleading. In the unripe organs the morphological precursors of sperm occur, consisting of the chemical precursors of the protamines. A microscopic control of the material under examination is recommended to ensure the ripeness of the testicles and the homogeneity of the spermatozoa. In many cases ripening does not set in in all parts of the organ at the same time.

Preparation of the Material for Investigation.

The first step is the isolation of the spermatozoa from the testicles. The testicles are put through a mincing machine (if necessary, several times) and the pulpy mass suspended in 4 or 5 volumes of water. In working up the testicles of the carp and closely allied species a solution of sodium sulphate (10 parts of the cold saturated solution to 90 parts water) is used instead of water. The suspension of the pulped testicles is well shaken in a shaking-machine and then worked through a sieve of coarse fabric (muslin) or wire with the help of a spatula. The milky liquid is treated with dilute acetic acid with constant stirring until strongly acid to congo red; the spermatozoa then separate as an easily filterable mass. A large excess of acetic acid must be avoided. The acetic acid precipitate is filtered through a pleated paper, washed with weak acetic acid and extracted with several lots of alcohol, first at room temperature and then at the boiling-

[1] Organs which have been preserved with salt are not suitable for the preparation of protamines (see under Histones, p. 66).

point. The last traces of a
evaporation of the ether a whit
be preserved in this state for a long
are now possible.

Method I. (Kossel, 77).—About 100 grams of the flour
shaken for half an hour with 500 c.c. 1 per cent. sulphuri
filtered. The extraction of the residue is repeated until a te
of the sulphuric acid extract no longer gives a definite pr
with alcohol. The extractions must be carried through in on
since on longer contact with sulphuric acid the nucleic acid begi
decompose, forming products which are troublesome. The sulphuric
acid extract is precipitated with three volumes of alcohol and the precipitate, consisting of the protamine sulphate, collected, dissolved in a little hot water and reprecipitated with alcohol. The precipitate obtained from 100 grams of the floury mass is dissolved in about 1½ litres of hot water and allowed to cool, when a small part of the sulphate separates as a yellow or brown oil. The supernatant liquid is separated from the least soluble part of the protamine sulphate, evaporated to a small volume and transferred to a separating funnel to collect the main bulk of the oil. Thus the middle fraction of oil is the purest.

Further purification is effected by treating a warm aqueous solution of the protamine sulphate with sodium picrate. The well-washed precipitate is freed from picric acid by shaking with toluene in the presence of an excess of sulphuric acid and the protamine sulphate precipitated from the sulphuric acid solution by alcohol. This alcohol precipitation is repeated once more. The consistency of the precipitate depends upon the acidity of the solution; if there is not sufficient acid present, a turbid solution results which can be flocculated by the cautious addition of sulphuric acid. If the precipitate is sticky, too much acid has been added. In this case it must be redissolved in water and reprecipitated with alcohol. The protamine sulphate should come down as a pure white powder. It is washed with alcohol and then with ether and dried in a desiccator. The yield from ripe herring testicles is from 15 to 20 per cent. of the dried sperm mass.

When larger quantities are worked up, alcohol can be saved by reducing the volume of the sulphuric acid extracts by evaporating down on the water bath, but these extracts contain small amounts of other extractives which are at once removed by the alcohol precipitation described above. During evaporation these substances are partly decomposed and contaminate the

preparation.[1] The sulphuric acid extracts are worked up as follows: The combined extracts are neutralised with baryta and evaporated on the water bath. The oil which separates is purified by means of the picrate as described above.

Most protamines can be prepared in a similar way. In the case of the protamines of carp sperm, however, the sulphate does not separate as an oil.

Method II.—This method is based upon the earlier communications of Schmiedberg (156), of Malenûk (123), and of Nelson-Gerhardt (133). It depends upon the fact that, on digestion with cupric chloride, the nucleic acids are converted into insoluble copper salts, while the protamines go into solution. The latter are precipitated as picrates and these are converted into alcohol precipitable sulphates by dissolving in dilute acetone and adding sulphuric acid.

The procedure is as follows: 100 grams of the dried sperm (p. 19) are digested with a solution of 100 grams of cupric chloride in a litre of water for three days in an incubator, the mixture being shaken at intervals. The precipitate is filtered off by suction, suspended in water and refiltered three times, and washed until the filtrate no longer gives an appreciable precipitate with concentrated sodium picrate solution.

The combined filtrate and washings are treated with a concentrated sodium picrate solution until the precipitate flocculates and sinks rapidly. It is filtered off, washed with very dilute sodium picrate and, while still moist, dissolved by gently warming in a mixture of 1 volume acetone and 3 volumes water. The solution is filtered and treated with half its volume of alcohol; 20 per cent. (by volume) sulphuric acid is then added drop by drop and with constant stirring until no further precipitate is formed. An excess of sulphuric acid makes the precipitate oily and dissolves it and must be avoided. The precipitate is filtered off and treated with absolute alcohol which makes it hard and friable. It is washed by decantation several times with alcohol, then with ether and filtered, washed with ether and dried in a desiccator.

For further purification of the preparation its resistance to peptic digestion may be used. A solution of 10 grams of protamine sulphate is digested for 24 hours at 37° with about 250 c.c. water containing 0·1 gram commercial pepsin and 0·5 gram hydrochloric acid. The digested liquid is neutralised with soda and the protamine precipitated

[1] It is possible to diminish the decomposition of the impurities by concentrating in vacuo, but the beginning of the distillation is accompanied by troublesome frothing.

as picrate, which is then converted to sulphate. This is redissolved and reprecipitated in the solid form as in Method I.

The second method of preparation is based upon experience gained with salmine. The first method is preferred to the copper method, although in certain cases, for example with the sperm of the carp, it does not give the oily precipitate of protamine. In certain cases protamine can be obtained by applying the copper method after the sperm has been extracted by the first method. The purification by peptic digestion can also be applied to a preparation obtained by Method I.

CHAPTER IV.

PROPERTIES AND COMPOSITION OF THE PROTAMINES.

As mentioned before, the protamines are only found in the sperm of fish. The composition and properties of the protamines of seventeen species, of which fifteen are teleostei, have been examined. The protamines may be considered to originate as a simplified re-arrangement of the protein molecule, which takes place in different ways in the different species. The peculiar property of all protamines is their marked basic character. The basic protein units predominate in the protamine molecule and are so linked as to leave amino-groups free and reactive. In the different fish, the three basic units, arginine, histidine, and lysine are present in different amounts. By this means the protamines are easily classified. In the first group are included those protamines in which the simplified protein molecule has been rebuilt without the inclusion of either histidine or lysine; arginine is thus the only basic unit present.

The second group is characterised by the presence of two bases. Since—so far as our present knowledge goes—there is no protamine which does not contain arginine, the second basic unit is either histidine or lysine.

The third group contains all three bases.

Further subdivisions can be made by taking into account the molecular proportions in which the units are combined in the protamines. The general idea is indicated by the following illustrations. Arginine is denoted by a, histidine by h, lysine by l, the sum of the monoamino-acid molecules by m, and the relative amounts of the individual molecules by a number appended to the letter. Thus, the formula a_2m should indicate that there is one monoamino-acid molecule to two arginine molecules and $(ahl)_2m$ that arginine, histidine, and lysine are contained in the molecule concerned and that the nitrogen of the monoamino-acids amounts to half of the total basic nitrogen. The quantitative relations of the bases amongst themselves is not taken into account.

The protamines are the terminal members of a progressive series which stretches from the typical proteins through the histones to the protamines. The histones, closely related to the typical proteins, are formed in the sperm of some families of fish, while in that of others are found the widely different protamines. It is thus only to be expected that intermediate stages between the histones and protamines will be found in yet other families or species. This is actually the case. Basic proteins, whose position in the system has not yet been clearly established have been obtained. These are discussed at the end of the chapter.

Since the protamines are transformation products of high molecular weight of the typical proteins, a simple and definite structure cannot be given to them. The agreement with the formulæ given is in many cases not close. This is partly due to the methods of purification not having yet been sufficiently developed, and partly to the great difficulty of preparing an adequate quantity of raw material.

In a later chapter it will be shown that the conversion of the original neutral or acid proteins into basic derivatives can proceed in two directions. By development in one direction protamines are formed in which the basic nature is due to the free amino-group of guanidine. The protamines of the salmine group are the final products of this series. In another direction the conversion of the original proteins proceeds so that at least one of the two amino groups of lysine is developed as the amino group determining the character of the whole molecule. This is the case in the protamines of the sperm of the carp and related species. This important point is not expressed in the following scheme which only considers the results of chemical analyses.

The names used in the following table and also in the text are only intended to indicate the origin of the protamines and do not indicate any identity with other protamines.

CLASSIFICATION OF THE PROTAMINES.

I. Monoprotamines.

These contain arginine as the only basic constituent.

(*a*) First Sub-group, a_2m to a_4m.

"*Salmine Group.*"

Salmine, coregonine, truttine, salveline, clupeine, scrombrine, esocine, alalongine, thynnine, ancylodine.

(*b*) Second Sub-group, am_2.

Cyclopterine.

II. Diprotamines.

These contain two basic constituents.

(*a*) First Sub-group.

Arginine and histidine as bases.

"*Percine Group*" $(ah)_2m$.

(*b*) Second Sub-group.

Arginine and lysine as bases.

"*Cyprinine Group*" $(al)m_2$.

Crenilabrine, cyprinine, barbine.

III. Triprotamine.

These contain arginine, histidine, and lysine.

"*Sturine*" $(ahl)_2m$.

The proportion of monoamino-acids has so far only been ascertained in a few cases (see salmine and clupeine).

The following are now known as general properties of all protamines:—

(1) An alkaline reaction in aqueous solution.

(2) Formation of salts: the salts of those acids which form precipitates with proteins (e.g. ferrocyanic, phosphotungstic, and picric acids) are generally sparingly soluble.

(3) Formation of compounds with oxides and salts of heavy metals. Of these, the copper compound which gives rise to the biuret reaction is especially important.

(4) Sakaguchi's (152) reaction with sodium hydroxide, α-naphthol, and sodium hypochlorite.

(5) Colour reaction with triketohydrindenehydrate (ninhydrin reaction).

(6) Colloidal character of the aqueous solutions.

(7) Lævorotation of protamine salts in aqueous solution.

(8) Hydrolysis by trypsin and resistance to pepsin and erepsin.

I. Monoprotamines.

Sub-group Ia: "*Salmine Group*" ($a_2m - a_3m$).

SALMINE.

Salmine was found in the ripe sperm of the Rhine salmon (Salmo salar) by Miescher (127) and investigated by Miescher (127), Piccard (142), Kossel (81, 86), Dakin (82, 83), and by Goto (48).

A substance very probably identical with salmine was isolated from Californian salmon (Oncorhynchus Tschawytscha) by Taylor and analysed by him and by Kossel (100). A similar body was found by Kossel (100) in the testicles of another Salmonida, Coregonus albus (American "Whitefish"), and by Kossel and Staudt (106) in Coregonus macrophthalmus (Nüsslin) (the "Gangfisch" of Lake Constance). The identity of salmine with the protamine "salvelino" prepared by Kossel (100) from the sperm of Salvelinus (Cristovomer) Namaycush (American "Lake Trout") is still doubtful. Nor is there yet any reliable evidence that the protamine "Esocine" (100) occurring in the sperm of the pike is identical with salmine. The arginine content of both salvelino and esocine is the same as that of salmine, but the specific rotations are different.

Since Miescher's early analyses there have been many attempts to find out the formula of salmine. Both the platinichloride and the sulphate have been employed. Miescher (127) first put forward the formula $C_{15}H_{30}N_8O_3$; Schmiedberg (129) suggested $C_{15}H_{28}N_8O_2$; Kossel (77) gave the formula $C_{30}H_{57}N_{17}O_6$. Goto's analyses also fitted in with these formulæ. Later, Kossel and Dakin's (83) investigations on the hydrolysis products showed that the molecular formula must be much higher. They gave the following values for the proportional amounts of the hydrolysis products of salmine:—

	Per cent.
Arginine nitrogen	89·2
Serine nitrogen	3·25
Aminovaleric acid nitrogen	1·65
Proline nitrogen	4·3
Loss	1·6

Various molecular proportions can be calculated from these values which are in agreement with those of Piccard, Goto, and others. For example, the values would correspond with 10 molecules arginine, 2 molecules serine, 2 molecules proline, and 1 molecule aminovaleric acid, which would give a formula $(C_{42}H_{78}N_{16}O_{12})_x$, and a molecular weight of at least 2025. The values would also fit the formula

$(C_{22}H_{42}N_{12}O_3)_x$ which would correspond to 12 molecules arginine, 2 molecules serine, 1 molecule aminovaleric acid, and 3 molecules proline.

Taylor analysed the salmine obtained from Oncorhynchus in the same way and arrived at the formula $C_{30}H_{57}N_{15}O_{12}$. He suggested that the molecule contained 12 molecules arginine, 3 molecules serine, 1 molecule aminovaleric acid, and 2 molecules proline (182).

The difficulty of completely hydrolysing such complex substances makes it impossible to distinguish between the possible formulæ by the present analytical methods.

As the free base, salmine has been little investigated.

Salmine Sulphate.—This salt has been prepared by the methods described above (p. 18 *et seq.*). 100 parts of water at room temperature dissolve 1·27 parts of the salt. It is more soluble in the presence of excess of sulphuric acid (77). It is easily soluble in hot water from which it separates on cooling as a colourless oil if the solution is sufficiently concentrated.

A slight turbidity is produced if 5·5 c.c. of saturated ammonium sulphate are added to a mixture of 2 c.c. of a 2 per cent. solution of salmine sulphate and 2·5 c.c. of water. The precipitation of the oil is complete if 7·5 c.c. of saturated ammonium sulphate is added to 2·5 c.c. of the salmine sulphate solution (Goto, 48).

Ether, or a few drops of alcohol or acetone, assists the separation of the oil. In the presence of large amounts of alcohol the sulphate comes down as a powder.

The refractive index of the oil precipitated from the solution is 1·442 (77).

The specific rotation of salmine sulphate is $[\alpha]_D = -80{\cdot}97°$ (Kossel, 77). The salmine (coregonine) from Coregonus macrophthalmus had $[\alpha]_D = -81{\cdot}05°$ (106),[1] (Kossel and Staudt). In the presence of dilute sulphuric acid the specific rotation diminishes even at room temperature. This is obviously due to hydrolysis.

Salmine Hydrochloride is readily soluble in water and can be precipitated as an oil from a hydrochloric acid extract of dried sperm by addition of sodium chloride.

Salmine Carbonate is also readily soluble.

Salmine Platinichloride.—This salt is especially suitable for analysis. It can be prepared by adding the requisite amount of barium chloride (avoiding an excess) to a solution of the sulphate and evaporating the

[1] Gawrilow's values (100) for salmine and other protamines are distinctly different.

solution of the hydrochloride which must not contain an excess of acid. The residue is dissolved in methyl alcohol with the addition of a drop of concentrated hydrochloric acid and precipitated with ether. The precipitate is redissolved in methyl alcohol and reprecipitated with ether three times. The precipitate of hydrochloride is completely dissolved in dry methyl alcohol and a dilute freshly prepared solution of platinic chloride in dry methyl alcohol added with constant stirring. The platinum salt is precipitated as a powder. The liquid is decanted off and a little more methyl alcoholic platinic chloride added; after standing a day the precipitate is filtered off, washed with methyl alcohol and ether and dried in a desiccator. This method (Goto, 48) has advantages over the earlier one of Miescher and Piccard in which the salt was precipitated from aqueous solution.

Analyses of the salt by Piccard and Goto corresponded with the formula $C_{30}H_{57}N_{17}O_6 . 4HCl . 2PtCl_4$, which, as stated above, cannot be regarded as the true empirical formula of salmine.

Salmine Nucleate.—The salt-like compound found by Miescher in the sperm of the salmon is especially interesting physiologically. According to Miescher there are 35·56 parts of protamine to 60·50 parts by weight of nucleic acid in the heads of the spermatozoa after exhaustive extraction with alcohol and ether, and this should represent the composition of a "neutral protamine nucleate." These numbers depend upon the formula which is used as the basis of the calculation. Burian (14A) selected another formula and arrived at the numbers 59·83 per cent. nucleic acid and 35·32 per cent. protamine. In his early investigations Miescher made the important observation that an aqueous solution of nucleic acid gave a precipitate with salmine which is very similar to the salt-like compound of the two substances present in the spermatozoa heads. This compound with clupeine was later further examined by Steudel (see below).

Other Salts.—Salmine salts are precipitated from acid solution by phosphotungstic acid, tungstic acid, picric acid, flavianic acid, chromic acid, hydroferrocyanic acid, and the other protein precipitants. Silver nitrate and sulphate form compounds with salmine salts which are not decomposed by excess of baryta, and in this respect resemble the compounds of arginine and histidine used for quantitative analysis. The mercury and cuprous compounds are very sparingly soluble or insoluble (5). A salt of the latter is precipitated, if a solution of salmine sulphate is treated with copper sulphate and sodium bisulphite. If copper hydroxide is added to a solution of the free base, the metal hydroxide dissolves with the formation of a violet colour (cf. clupeine).

Compounds with Typical Proteins.—If an ammoniacal solution of salmine is added to a protein solution, a compound of the protein with salmine is precipitated (Kossel, 76). This reaction can be observed with most protamines and has been examined in more detail in the case of clupeine.

Salmine is precipitated with hydroferrocyanic acid. This reagent separates crude clupeine and crude cyprinine into several fractions, but with salmine it gives a single precipitate (Schenck, 154A).

As previously mentioned, protamines have been found in the sperm of other Salmonidæ which can be considered as identical with the salmine from Rhine salmon, while there are others whose identity with salmine is still doubtful. Among the former is the substance from Coregonus macrophthalmus which has been shown to have the same content of arginine and the same specific rotation (p. 26), probably also the protamine from Oncorhynchus Tschawytscha, and from Trutta fario. Among the latter are the little-known substances from Coregonus albus and Salvelinus (Cristovomer) Namaycush.

The percentage of arginine nitrogen is as follows :—

Substance from—	I.	II.	
1. Oncorhynchus Tschawytscha	—	86·2	— Taylor, 1908 (182).
2. Coregonus albus . .	—	87·3	— Kossel, 1913 (100).
3. „ macrophthalmus .	—	90·6	93·37 Kossel and Staudt, 1926 (106).
4. Salvelinus Namaycush .	—	88·9	— Kossel, 1913 (100).
5. Salmo Salar . . .	89·3	89·2	— Kossel and Gross, 1924 (104).
6. Trutta fario	—	88·14	91·07 Kossel and Schenck 1927.

The figures of (3), (5), and (6) were determined by the flavianic acid method, the rest by the older silver-baryta process. (5) was hydrolysed with hydrochloric acid. In column I. the total nitrogen was determined *before* removal of the sulphuric acid (A, p. 6), and in column II. *after* removal of sulphuric acid (B, p. 6).

CLUPEINE.

Clupeine has so far been found only in the sperm of the herring, and has been more extensively examined than any of the other protamines since the raw material is the most accessible. The clupeine first prepared by Kossel from the testicles of the herring was originally regarded as identical with salmine on account of the analysis of the

sulphate, the solubility, the specific rotation and the refractive index. Later it was proved that these two protamines were different. The most conclusive proof was the finding of a unit in clupeine which is not present in salmine; this is alanine (Kossel and Dakin, 83). According to Edlbacher the two substances behave differently towards dimethyl sulphate. Edlbacher estimated how many methyl groups combined with each 100 atoms of nitrogen when various proteins were treated exhaustively with dimethyl sulphate in alkaline solution. He found that the ratio $N : CH_3$ in clupeine sulphate was 100 : 24·4, in salmine sulphate from Rhine salmon 100 : 9·7, and from Oncorhynchus 100 : 8·9 (25).

From his analysis of clupeine sulphate Kossel calculated the formula $C_{30}H_{57}N_{17}O_6$ (77), which agrees with Piccard's analysis of salmine. On the basis of his analysis of the platinum salt Goto put forward the formula $C_{32}H_{62}N_{14}O_8$, which, of course, like all formulæ for protamines, only represents the simplest expression of the composition. Goto not only concluded from his analyses that salmine and clupeine were different but also obtained values which cast doubt upon the homogeneity of the clupeine preparation. Goto based his doubt on the fact that he found differences in the ratio of carbon to nitrogen in the platinum salt and copper salt of clupeine. This is shown by the following values:—

	$\frac{\text{Weight of carbon}}{\text{Weight of nitrogen}}$
In clupeine sulphate (Kossel) . . .	1·51
In clupeine copper sulphate (Goto) . . .	1·55
In clupeine platinichloride (Goto) . . .	1·81
In salmine platinichloride (Goto) . . .	1·55

Investigations by Kossel and Schenck led to the same result. It appeared that the clupeine sulphate prepared by the above method contained several protamines, which could be separated by precipitation with hydroferrocyanic acid and sulphosalicylic acid and by the solubility of their picrates in acetone. The values for the arginine nitrogen expressed as a percentage of the total nitrogen were as follows:—

	I.	II.
Fraction 4	77·24	82·35
Fraction 5	91·48	93·38
Fraction 6	88·11	94·26
	88·82	94·88

Column I. is calculated from the total nitrogen determined before removal of sulphuric acid and column II. after removal of the sulphuric acid with baryta (p. 6). The data for clupeine therefore probably mainly refer to a mixture of these fractions.[1]

Hydrolysis of clupeine has shown the presence of alanine (83), serine (82), an aminovaleric acid (79), and proline (83), as well as arginine. The relative proportions of the nitrogen of arginine, of alcohol-soluble substance, and of the alcohol-insoluble amino-acid mixture are approximately the same as those of salmine. The values found for the amino-acids approximate to a mixture of 2 molecules aminovaleric acid, 1 molecule serine, and 1 molecule of alanine besides proline (83).

Ellinghaus found that 1 gram of dry clupeine had a calorific value of 5637 calories (30A).

Clupeine can be obtained as the free base by treating the aqueous solution of the sulphate with baryta. The alkalinity of free clupeine is as great as the arginine contained in it, i.e. for every nine nitrogen atoms in clupeine two represent one basic equivalent.

Clupeine Sulphate is very similar to salmine sulphate. It contains $2H_2SO_4$ to seventeen atoms of nitrogen. It is easily soluble in hot water and separates from the solution on cooling as a clear colourless oil. The data for the solubility in cold water do not agree owing to insufficient attention being paid to the temperature. The oil precipitated at room temperature contains about 50 per cent. of water, has a refractive index 1·4430 (76)—1·439 according to Kurajeff (110)—and becomes turbid on cooling since drops separate. If it is allowed to dry, an amorphous easily powdered residue is obtained. If clupeine sulphate is dissolved in hot water and the oil, which separates at ordinary temperature, removed, the resulting solution contains 1·29 per cent. clupeine sulphate—according to Kurajeff 1·62 per cent. Thus, at room temperature clupeine sulphate is soluble in 62 to 77 parts of water. It is precipitated from this solution, like salmine, in the solid form by large quantities of alcohol and as an oil by a little alcohol, acetone or sodium chloride.

Kossel gives the specific rotation of clupeine sulphate as $[\alpha]_D = -83{\cdot}07°$, Kurajeff (110) as $[\alpha]_D = -85{\cdot}49°$, Waldschmidt-Leitz, Schäffner, and Grassmann (193) as $[\alpha]_D = -84°$.

[1] It must be borne in mind that the method of preparation of the sulphate described above gives a fractionation since the sparingly soluble as well as the most soluble part of the oil is separated and only the middle fraction is used for examination.

Goto prepared a *clupeine copper sulphate* by boiling a solution of clupeine sulphate with copper hydroxide and precipitating the concentrated violet solution by adding alcohol. The salt thus obtained contained little copper. Violet compounds rich in copper are formed if a solution of the free protamine is boiled with copper hydroxide. These compounds belong to the class of the complex salts which are produced in the biuret reaction with proteins (48).

The Hydrochloride, Carbonate, and Nitrate of clupeine are readily soluble in water. The hydrochloride is extracted from the dried sperm and can be precipitated as an oil from this solution by salt. The hydrochloride diffuses through parchment paper, but the sulphate does not. Goto prepared *clupeine platinichloride* by the method described for salmine (p. 26) and found that the simplest expression of the analytical results was $C_{30}H_{62}N_{14}O_5$, 4HCl, $2PtCl_4$. Kurajeff found that *clupeine chromate* contained $2H_2CrO_4$ for every seventeen atoms of nitrogen (110).

Clupeine salts give precipitates with the reagents mentioned for salmine. The compounds with silver, mercury, and copper hydroxide are also very sparingly soluble.

Sparingly soluble salts of clupeine with organic acids have been prepared and analysed by Steudel and his collaborators. Steudel and Peiser (177A) showed that clupeine-eosine contained 59·81 per cent. eosine and 40·19 per cent. clupeine; analyses by Mandel and Steudel (122A) showed that clupeine-germanine consisted of 56·28 per cent. germanine and 43·74 per cent. clupeine.

The Compounds of Clupeine with Nucleic Acid are of special interest. Steudel and Peiser (177A) found 53·33 per cent. guanylic acid and 46·67 per cent. clupeine in the guanylic acid salt, and 61·15 per cent. yeast-nucleic acid, and 38·85 per cent. clupeine in the yeast-nucleic acid salt. If sodium thymonucleate, the sodium salt of the acid from fish sperm, is added to a solution of clupeine sulphate, a precipitate of clupeine nucleate is formed just as with salmine. If the two components of this salt are added in equivalent quantities, the precipitate consists of a neutral salt of clupeine and nucleic acid. Steudel's analysis of this salt gave the ratio of phosphorus to nitrogen as 1 : 3·211. The work of Mlescher (129) on salmon and of Mathews (124), and Steudel (172, 173, 174) on herring showed that the sperm heads of these fish after extraction with water, alcohol, and ether consisted almost entirely of protamine nucleate. Steudel found for the sperm heads the ratio P : N = 1 : 3·237, which closely agrees with the value obtained from the artificially

prepared salt. The composition of the sperm heads (after drying and extraction with alcohol and ether) is accordingly 73·5 per cent. thymonucleic acid and 26·5 per cent. clupeine. Lynch (121A) found about 70 per cent. nucleic acid and 30 per cent. coregonine in Coregonus albus and gave $C_{80}H_{130}N_{34}O_{28}(C_{15}H_{28}N_{12}P_4O_{24})_4$ as the probable formula of the "chromatin" of the sperm head. The chemical constituents are thus built up in the living organ in the same proportions as when they are obtained by combining artificially nucleic acid and protamine. But this similarity in composition does not exclude the presence in the living organ of molecular aggregates which are absent in the artificial product. With reference to this point Steudel compared his clupeine nucleic acid with the sperm heads of the herring. Differences were found in the extent of swelling, in polarimetric behaviour and in viscosity. These observations indicate that in the sperm heads, dissolved in sodium hydroxide, a certain inter-molecular structure is retained which disappears on prolonged action of the reagent.

The calorific value of clupeine nucleate in the sperm heads is just as great as that of the artificial product, i.e. one gram of nucleate gives 4400 calories. This number agrees with the calorific value of a mixture of the two components in the proportions indicated by the analyses (Ellinghaus, 30A).

Compounds of clupeine with the higher proteins are formed as precipitates when proteins are added to an aqueous solution of clupeine under suitable conditions. The formation of these precipitates was first observed by Kossel (75) and then examined by Hunter (69) and Beth af Uggias (118). These precipitates are formed with casein, egg albumin, hemielastin, gelatin, edestin, haemoglobin, and heteroalbumose but not with elastin peptone, deuteroalbumose, and histopeptone; nor can they be formed with a series of polypeptides. The two last-named authors tried to find the proportions in which the constituents were combined. In each case it had to be decided whether a chemical compound of the two proteins was formed or whether the process was one of absorption. According to B. af Uggias it is very probable that, with clupeine-haemoglobin and clupeine-casein, compounds of definite composition are formed. 100 parts of the compound contain 95 parts of haemoglobin and 5 parts clupeine, if sufficient or an excess of haemoglobin is added for the quantitative precipitation of the clupeine. If, however, clupeine is in excess, a precipitate with a greater protamine content is obtained. B. af Uggias obtained similar values with casein, while Hunter found the clupeine nitrogen to be 40 per cent. of the total nitrogen which corresponds to a composition of about 75 parts casein to 25 parts clupeine.

The clupeine protein compounds in most cases are only formed if the free base clupeine is added to the protein; but with casein the compound is formed if the two components as salts are mixed in neutral solution. The compounds are decomposed by pepsin-HCl, the non-basic protein being hydrolysed by the enzyme, while the clupeine remains unattacked, and can be obtained from the solution in pure condition by means of sodium picrate. Hunter made use of this behaviour for the quantitative estimation of clupeine contained in the compound.

SCOMBRINE.

Scombrine was first prepared by Kurajeff (110) from the testicles of the mackerel (Scomber scomber) of the Baltic Sea by the methods described above (p. 18). By analysis of the sulphate and chromate he arrived at the formula $C_{30}H_{62}N_{16}O_8$, whilst Goto (48) by analysing the platinum salt found values corresponding to the formula $C_{22}H_{72}N_{16}O_2$.

Besides arginine only proline and alanine (85) have been found among the hydrolysis products, but it is probable that a third monoamino-acid accompanies alanine in the alcohol insoluble part of the monoamino-acid fraction. The quantitative relations are as follows:—

	Percentage of Total Nitrogen.
Arginine	88·8
In alcohol-insoluble (alanine + unknown substance) .	6·8
In alcohol-soluble (proline)	3·8
Loss	0·6

The absence of serine distinguishes scombrine from salmine and clupeine. A further difference is that scombrine under the conditions employed by Edlbacher does not methylate with dimethyl sulphate (25).

Scombrine Sulphate scarcely differs in properties from the sulphates of the protamines already described. According to Kurajeff it contains $2H_2SO_4$ to 16 N. He states that the liquid left after the separation of the oil at room temperature contains 2·2 per cent. of scombrine sulphate, i.e. 1 part sulphate dissolves in 45·5 parts of water. The refractive index of the oil is 1·436 and the specific rotation of the sulphate in aqueous solution is $[\alpha]_D = -71{\cdot}81°$.

Data for the hydrochloride are not available, but the platinum salt was analysed by Goto (48) giving the formula—

$$C_{22}H_{72}N_{16}O_2 . 4HCl . 2PtCl_4.$$

Kurajeff found that the chromate contained $2H_2CrO_4$ to 16 N.

ESOCINE.

Esocine was first obtained from the testicles of the pike by Hunter (69) and later by Kossel (100). It does not differ in properties from the protamines of the salmine group. It contained 86·3 per cent. of arginine nitrogen.

THYNNINE.

So far only one compound containing tyrosine has been found among the protamines of the salmine group. This is thynnine. (Possibly also the little-known xiphiine.)

Thynnine is present in the ripe testicles of the tunney fish (Thynnus Thynnus). The basic protein in these organs was first described by Ulpiani (189) and later by Dezani (22). Ulpiani prepared the compound in 1902 by extraction of the testicles and precipitation of the sulphate as oil by alcohol according to Kossel's method. He modified the method, however, by precipitating the base with ammonia during the process. From analyses of the sulphate, carbonate, molybdate, and tungstate he obtained the following formulæ :—

$$C_{30}H_{114}N_{30}O_8(SO_4)_2 . 4H_2O.$$
$$C_{30}H_{114}N_{30}O_8(CO_3)_2 . 13H_2O.$$
$$C_{30}H_{114}N_{30}O_8(Mo_7O_{24})_4 . 15H_2O.$$
$$C_{30}H_{104}N_{30}O_8(Wo_2O_7).$$

He separated arginine from the hydrolysis products and showed that there was yet another base which was not further characterised. He came to the conclusion that the compound prepared from the sperm of the tunney fish should be classed with the histones although it showed several variations from the histone type.

Dezani (1908) found histidine and lysine among the hydrolysis products besides arginine. He also found ammonia.

According to Kossel (100) the sperm of the tunney fish contains a protamine, thynnine, belonging to the salmine group. The composition is as follows :—

	Percentage of Total Nitrogen.
Ammonia	—
Histidine	—
Arginine	79·5
Lysine	—
Monoamino-acids	21·0
Tyrosine in above	0·6

From Ulpiani's data there is no doubt that the compound prepared by him is identical with thynnine.

ANCYLODINE.

This protamine was prepared by Staudt from the testicles of Sagenichthys ancylodon (South America).

The sulphate is sparingly soluble in cold water at neutral reaction and separates from the supersaturated solution as an oil. Two estimations gave the arginine nitrogen as 77·67 and 74·66 per cent. (Calc II., p. 12).

The solution gives a biuret and Sakaguchi reaction, but no colour with Millon's reagent, diazobenzenesulphonic acid, or Hopkins and Cole's tryptophan reagent. Precipitates are formed with potassium ferrocyanide and acetic acid, sulphosalicylic acid, picric acid, and with ammoniacal Witte's peptone solution. The substance is therefore regarded as a member of the salmine group, but contains less arginine than is expressed by the formula a_9m.

Sub-group Ia (am_2).

CYCLOPTERINE.

At present only one member of this group is known—cyclopterine. It was first prepared by Morkowin (131) from the testicles of Cyclopterus lumpus (from the Baltic Sea). His analysis of the sulphate gave 42 per cent. C, 6·73 per cent. H, 22·37 per cent. N, 8·10 per cent. S, and the compound possessed the properties of the protamines.

Kossel and Kutscher (80) examined the hydrolysis products and found :—

	Percentage of Total Nitrogen.
Arginine N	67·7
Monoamino-acid N	29·9
Tyrosine in above	2·2

The large amount of tyrosine (more than 8 per cent. by weight) is remarkable, also the high value for nitrogenous substances absorbed on the barium sulphate precipitate, and the appearance of a tryptophan (Hopkins and Cole) reaction. The analyses were performed with a limited amount of material and require confirmation. The supply of material, however, is governed by chance.

II. Diprotamines.

Sub-group IIa $(ah)_2m$.

PERCINE.

Percine, the only member of this sub-group, has so far only been found in two species of the perch—Perca flavescens ("yellow perch") and Stizostedium vitreum ("pike perch")—both from North America. The preparations analysed by Kossel only differed in properties from those already described in giving reactions characteristic of histidine. The analyses gave :—

	Percentage of Total Nitrogen.
Arginine N	76·1
Histidine N	5·6
Monoamino N	9·8

Sub-group IIb.

Cyprinine Group.

CRENILABRINE.

The composition of crenilabrine from Crenilabrus Pavo (Mediterranean) is only known in rough detail. According to Kossel (94) it approximates to the formula alm_2 which can be derived from the following analysis :—

	Percentage of Total Nitrogen.
Arginine	42·3
Lysine	11·0
Monoamino-acids	25·1

The arginine nitrogen was determined by Kjeldahl's method. The amount of lysine was too small for weighing as picrate and could only be determined by a nitrogen estimation. In its general properties crenilabrine does not differ from the other protamines.

CYPRININE

(From Cyprinus Carpio.)

The sperm of the carp (and its nearest relations) contains products of a change which proceeds along two lines. One type of cyprinine is richer in arginine and poorer in lysine, while in the other type lysine

appears in excessive quantity and the percentage of arginine falls. These differences were detected by Kossel and Dakin (82) in their first analyses of the protamine from carp sperm in 1904. They showed that the two types of protamine contained arginine and lysine but not histidine, that their sulphates were not precipitated as oils from aqueous solution as is the case with the majority of protamines, but that otherwise they possessed the properties of the protamines. Analyses of two preparations are given in columns I. and III. :—

	Percentage of Total Nitrogen.	
	I.	III.
Arginine	8·7	28·0
Lysine	30·3	6·6

At the time the behaviour of these substances gave rise to the surmise that a mixture of several substances was present. This was confirmed by the later work of Kossel and Schenk (107). A separation of these types can be effected by precipitation with hydroferrocyanic acid, or sulphosalicylic acid, which give sparingly soluble salts with the bases rich in arginine, while the bases rich in lysine are not precipitated. In this way types were separated in which the arginine and lysine contents were as follows :—

	Percentage of Total Nitrogen.	
	Arginine-poor Type.	Arginine-rich Type.
Arginine	3·28	29·76
Lysine	45·56	10·80

Among the monoamino-acids aminovaleric acid was detected, thus confirming Kossel and Dakin, and in addition proline and alanine were found but not tyrosine (contrary to the finding of Kossel and Dakin).

A similar substance **barbine** (11·5 per cent. arginine nitrogen, 38·8 per cent. lysine nitrogen, and 12·8 per cent. proline nitrogen) was prepared from a small quantity of the testicles of the barbel.

III. Triprotamine.

STURINE $(ahl)_3m$.

Sturine was discovered by Kossel (76) in 1896 in the testicles of the German sturgeon from the Baltic Sea (Accipenser Sturio). Later, Kurajeff (111) and Malenfick (123) extracted protamines from the testicles of sturgeons from the Caspian Sea (Accipenser Guldenstädtii

and A. stellatus). It is not yet settled whether the protamines from the three species are identical.

Analysis of the sulphate of the sturine from Accipenser Sturio gave the formula $4C_{30}H_{60}N_{15}O_7 . 11H_2SO_4$ (Kossel, 77), whereas Goto (48) gave $C_{30}H_{62}N_{17}O_3 . 4HCl . 2PtCl_4$ for the platinum salt.

Kurajeff (111) found that the composition of the sulphate from Accipenser stellatus corresponded to $C_{30}H_{72}N_{18}O_3 . 4H_2SO_4$, and Malenčick (123) gave the formula $C_{30}H_{62}N_{16}O_7 . 2H_2SO_4$ to the sulphate prepared from Accipenser Guldenstädtii.

In these formulæ the C : N ratio varies. In Kossel's formula it is 1·65, in Goto's 1·71, in Malenčick's 1·78,[1] and in Kurajeff's 1·97.

On hydrolysis of sturine Kossel (77) found arginine, histidine, and also lysine. Later, Kossel and Dakin (85) were able to detect alanine and leucine (or an isomer). The absence of aminovaleric acid, serine and proline is remarkable.

Quantitative estimations of the hydrolysis products were carried out by Kossel and Kutscher (80) and repeated later by Kossel and Weiss (96) :—

	Percentage of Total Nitrogen.	
	Kossel and Kutscher. (1901).	Kossel and Weiss. (1914).
In the arginine fraction . .	63·5	67·4
„ histidine fraction . .	11·8	10·1
„ lysine fraction . .	8·4	7·5

The properties of sturine do not differ greatly from those of the protamines of the salmine type, but the sulphate is more readily soluble. The separation of the oil occurs at a greater concentration of the aqueous solution. No oil separates even from a 10 per cent. solution of sturine sulphate. Kurajeff also mentions the greater solubility of the protamine sulphate from Accipenser stellatus. The separation can be brought about by the help of a small quantity of ether, acetone or alcohol. Ammonia causes precipitation if the solution is not too dilute. Salt causes precipitation but less readily than with the protamines of the salmine group.

In two experiments Goto (48) found the specific rotation $[\alpha]_D = -60°$, and $-58·8°$ (probably for the sulphate).

[1] Malenčick made the entirely groundless assumption that the difference in the nitrogen values was caused by admixture with adenine; cf. *Zeit. physiol. Chem.*, 69, 138 (1910).

TRANSITION FORMS.

As mentioned above (p. 23) the sperms of fish contain on the one hand histones, which have the character of the higher proteins; on the other hand, protamines which have a simpler structure and are the final stage in the process of degradation. It can thus be readily understood that types may occur which are intermediate between the two extremes, or that an extract may contain a mixture of the two. Such conditions are seen especially in the organ in the unripe state (see later under Histones).

A protamine-like body, which cannot be classified in the above scheme, was prepared by Dunn (24A) from the testicles of Sardinia coerulea. The raw material was collected during the spawning season off the Californian coast. The finely disintegrated testicles were extracted directly with 1 per cent. sulphuric acid, and the extract precipitated with alcohol and purified by Kossel's method. The distribution of nitrogen in the products of hydrolysis by hydrochloric acid was estimated by Dunn using van Slyke's method and the colorimetric method of Folin and Looney (43A).

	Percentage of Total Nitrogen.
Amide nitrogen (ammonia)	0·86
Humin „	2·83
Arginine „	27·83
Cystine „	0·60
Histidine „	23·02
Lysine „	5·48
Amino „ of filtrate	25·89
Non-amino „ „ „	14·33

1·09 per cent. tyrosine and 0·87 per cent. tryptophan were also detected. The high histidine content, which has as yet only been revealed by the differential method of van Slyke, is very remarkable.

CHAPTER V.

DECOMPOSITION AND CONSTITUTION OF THE PROTAMINES.

So far conceptions of the structure of the protein molecule have been based upon results obtained by hydrolysis. They suggest lines for attacking the problem by other methods: synthesis, reduction, and oxidation.

The hydrolysis of the protamines has been carried out just as with the other proteins by acids or alkalis at high temperatures, or by enzymes. By grading the concentration of the acid or alkali, the degree of temperature and the time of action, the reaction can be more or less widely extended to give the intermediate products of hydrolysis.

Gross (55), in order rapidly to stop the action of the sulphuric acid during the hydrolysis of protamines at high temperatures, constructed a special autoclave which made it possible to control the time of heating accurately and used it for obtaining intermediate products of the reaction.

Behaviour of Protamines with Proteolytic Enzymes.

Investigation of the action of proteolytic ferments on the protamines has made an important addition to our knowledge of the mode of action of the ferments and at the same time of the constitution of the protamines. It is quite clear that light can only be thrown upon the nature of enzyme action if the substrates to be examined are of definite composition and their structure is known as far as possible. Only then can the question be investigated of the point of attack of the various enzymes and the influence of other atomic groups contained in the molecule. From this point of view the protamines have a definite advantage as substrates for enzyme action over the more complexly constituted proteins. On the other hand, there is the advantage that the specific enzyme splits off the members of the protamine more delicately than is possible by the cruder method of acid hydrolysis.

Kossel and Matthews (78) in 1898 discovered that salmine and

sturine were not hydrolysed by pepsin-HCl, but were hydrolysed by trypsin. These results were confirmed by Rogozinski (149) in 1912.[1] The action of the individual tryptic enzyme could only be made clear after the sharp separation of the various members of this group had been made possible by the methods devised by Willstätter and his school. By employing these methods which provided a means of separating trypsin and erepsin and using a new method of titration (see below) for following the degree of hydrolysis, Waldschmidt-Leitz and Harteneck (191, 192) obtained the results given in the following table which shows the behaviour of clupeine compared with that of the higher proteins. The action of pepsin is also shown.

SPECIFICITY OF PANCREATIC TRYPSIN AND EREPSIN.
(Protamine compared to the higher proteins.)

(− = No detectable hydrolysis, + = Hydrolysis, ++ = Vigorous hydrolysis.)

Substrate.	Enzyme.			
	Pepsin.	Erepsin.	Trypsin.	Trypsin-Kinase.
Alanylglycine	−	+	−	−
Leucylglycylglycine	−	+	−	−
(Examples of di- and tripeptides)				
Peptone	−+[2]	−	+	++
Clupeine	−	−	+	++
Thymushistone	+	−	+	++
Fibrin	++	−	−	+
Casein	++	−	−	+
(Examples of higher proteins)				

This table shows that—

(1) Di- and tripeptides are hydrolysed by erepsin.

(2) Clupeine, histone, and peptone are only hydrolysed by trypsin (activated or not activated).

(3) Higher proteins are only hydrolysed by trypsin-kinase.

The hydrolysis of clupeine was followed quantitatively by Waldschmidt-Leitz, Schäffner, and Grassmann (193) who found " that the

[1] Takamura's experiments (1814) in 1909 did not lead to any definite result—probably because the methods were not suitable for these experiments.

[2] Peptone is not a homogeneous substance. One peptone may be attacked by pepsin, another may not.

action of the separate enzymes ceases at a certain definite condition and that these conditions are related, by the formation of recognisable chemical groups, in simple numerical proportions."

This is shown by the following series of experiments :—

HYDROLYSIS BY TRYPSIN, TRYPSIN-KINASE, AND EREPSIN. SEQUENCE AND ACTION.

Experiment No.	Sequence of Enzymes.	Ratio of Action.
1	Trypsin	1
	Trypsin-kinase	3
	Intestinal erepsin	1
2	Trypsin	1
	Intestinal erepsin	1
	Trypsin-kinase	1
	Intestinal erepsin	2
3	Trypsin-kinase	2
	Intestinal erepsin	1

In these experiments the enzymatic hydrolysis was estimated on the basis of the appearance of free amino and carboxyl groups.

Either Sörensen's formol titration or van Slyke's method can be used for the estimation of the liberated amino groups. The determination of the carboxyl groups by the method of Willstätter and Waldschmidt-Leitz (197) depends upon the fact that in amino-acids, peptides and proteins only carboxyl groups react in alcoholic solution and can be estimated by titration. By ascertaining the acidity in 50 per cent. and 90 per cent. alcoholic solution it is possible to distinguish between the acidity due to free amino-acids and that due to peptide carboxyl groups.

Certain numerically sharply defined linkages within the clupeine molecule are broken by the individual enzymes. Experiments 1 and 2 illustrate the division of the compounds, which are hydrolysed by enzymes, into five groups, and experiment 3 into three groups.

Experiments 1 and 2 also show that with trypsin one-fifth of the total enzyme action is developed. The HCN activated plant enzyme papain acts in a similar manner.

From these experiments based upon the estimation of the liberated amino and carboxyl groups Waldschmidt-Leitz, Schäffner, and Grassmann conclude that the whole process of hydrolytic cleavage consists in the *breaking of peptide-linkages*, but that besides those which can be broken by enzymes there are also present peptide-linkages which are unattacked. In the structure of the latter the presence of a tertiary linking of proline to carboxyl is characteristic.

The differences in structure of the protamines are also shown by their behaviour towards enzymes. Waldschmidt-Leitz and Kollmann (194A) in quantitative experiments with four different protamines found a difference between the monoprotamines (clupeine, salmine, and scombrine) and the triprotamine sturine.

ENZYMATIC CLEAVAGE OF PROTAMINES.

(Symbols as above, p. 41.)

Preparation.	Enzyme.				
	Trypsin.	Trypsin-kinase.	Erepsin.	Papain.	Papain-HCN.
Clupeine sulphate .	+	++	—	—	+
Salmine sulphate .	+	++	—	—	+
Scombrine sulphate	+	++	—	—	+
Sturine sulphate .	—	+	—	—	+

Sturine in contrast to the protamines of the salmine group is not attacked by kinase-free trypsin. Greater differences are revealed if the action of these enzymes is followed quantitatively.

HYDROLYSIS OF PROTAMINES BY TRYPSIN, TRYPSIN-KINASE, AND PAPAIN-HCN, AND ENZYME ACTION.

(Waldschmidt-Leitz and Kollmann.)

(Data indicate the increase in acidity in c.c. 0·1N alkali for the hydrolysis of 0·142 gram protamine base. Thus the amount of nitrogen in each will be approximately the same. Hydrolysis was continued until values were constant.)

Protamine.	Enzyme.		
	Trypsin.	Trypsin-Kinase.	Papain-HCN.
Clupeine . .	0·90	3·10	0·94
Salmine . .	1·00	3·18	0·88
Scombrine .	0·60	2·96	0·78
Sturine . .	0·02	3·95	—

According to these values clupeine and salmine behave very similarly with all three enzymes, but differ from scombrine in the amount of decomposition by trypsin. As already stated (p. 42) the proportion of carboxyl in clupeine set free by trypsin is one-fifth of the total carboxyl which can be liberated by enzymes, while in scombrine it is only one-ninth (Zeigler, according to Waldschmidt Leitz and Kollmann). Sturine is not attacked.

So far the following have been isolated as products of the hydrolysis of protamines by enzymes :—

(1) Arginine by the hydrolysis of sturine by trypsin-kinase (Kossel, Staudt, and Waldschmidt-Leitz).

(2) Arginine, histidine, and lysine by the hydrolysis of sturine by impure "trypsin." In addition a substance was isolated by Kossel and Mathews (78) from which a well-crystallised silver salt of composition $C_{10}H_{22}N_7O_2 . 4HNO_3 . 2AgNO_3$. was obtained. The formation of this substance is obviously dependent upon definite experimental conditions which are not known; it cannot be decided which tryptic enzyme was used for these experiments carried out in 1898.

Hydrolysis by Acids.

The units of the protamines are obtained as end products of hydrolysis by the intensive action of mineral acids. They have already been enumerated. The division of the protamines into their groups depends upon the quantitative relations of these units. Prior to the complete decomposition into these hydrolysis products, intermediate products are formed of which the general nature has been established but which have not yet been sharply characterised as chemical individuals.

During the acid hydrolysis of salmine three distinct stages can be recognised. In the *first* stage the original protamine is converted into protone. In this way the protamine loses its properties of precipitating protein in weak ammoniacal solution, and of forming an oily sulphate. It also loses its normal physiological action (see below), but it still gives the biuret reaction. This change is almost complete on warming for half an hour on the water bath with 10 per cent. sulphuric acid.

If a higher temperature is employed, a typical biuret reaction is no longer given. According to Gross there are present besides free monoamino-acids and their peptides, products which only contain about 3 per cent. of their nitrogen in the form of free amino-groups

(as detected by van Slyke's method or Sörensen titration) and which yield arginine on further hydrolysis. In properties they resemble the dioxypiperazines. This is the *second* stage of hydrolysis.

The *third* stage, the complete decomposition into monoamino-acids and arginine, is attained by prolonged hydrolysis.

The first and second stages have only been differentiated in the more recent investigations, especially by the work of Gross (54). The substances described as protones have probably often been a mixture of the two stages.

Some of these intermediate products of hydrolysis are closely related to the protamines. They are called protones and like the protamines are characterised by the names of the fish, e.g. clupeone, and sturone. They are readily recognised by giving the biuret reaction with sodium hydroxide and copper sulphate. On further hydrolysis other products which no longer give the biuret reaction are formed from the protones.

The Protones.

The protones can be prepared in the form of their sulphates by the method used by Kossel (77) and Goto (48) as follows: 5 grams of the protamine sulphate are dissolved in 40 c.c. of water and the solution treated with dilute sulphuric acid so that 100 c.c. contain 10 c.c. H_2SO_4. The protamine is decomposed by boiling for half an hour under a reflux condenser. The liquid, which remains colourless or light yellow, is then precipitated with ethyl alcohol. The precipitate is dissolved in water, and again precipitated with five to six times its volume of alcohol. The precipitation is repeated three times.[1] The product still gives most of the characteristic protamine precipitation reactions, but the precipitates are generally more readily soluble. As already mentioned a very strong biuret reaction is given. The basic character is still very definite.

Of the salts of the protones the picrolonate deserves especial mention since it occurs in crystalline form. Kossel and Weiss (90) prepared it by mixing a dilute solution of free clupeone with an alcoholic solution of picrolonic acid. A precipitate is formed which consists of microscopic tufts of needles which appear light in the dark field of crossed Nicols. The light tufts show a dark cross, the axes of which are parallel with the chief axes of the prism. The crystals can be recrystallised from dilute alcohol.

[1] For a further possible purification by the acetic and picric acid method, see p. 46.

By suspending the picrolonate in dilute sulphuric acid and extracting with ether the picrolonic acid is easily removed, leaving a solution of clupeone sulphate. The crystallisation does not necessarily ensure the homogeneity of the precipitate.

Goto (48) prepared the free base from clupeone sulphate for determination of the molecular weight in aqueous solution by the boiling and freezing-point methods. The former method gave 419, and the latter 424.

The protones are laevorotatory like the protamines. The change from protamine to protone causes a decrease in the specific rotation. Goto found the following values for $[\alpha]_D$:—

Free clupeone	$-22{\cdot}02°$
Clupeone sulphate	$-49{\cdot}11°$
Scombrone sulphate	$-41{\cdot}25°$
Sturone sulphate	$-22{\cdot}5°$

Clupeone.—Complete hydrolysis of clupeone gives the same arginine value as is found on hydrolysis of the original protamine. Comparative experiments of this kind were first performed by Goto (48) and then by Pringle (88). The latter in addition to analysing the whole precipitate of clupeone sulphate thrown down by alcohol attempted to separate this precipitate into several fractions.

The fractionation was based upon the observation that if a solution of arginine in acetic acid is treated with an alcoholic solution of picric acid, a precipitate is formed which is soluble in excess of picric acid. If protones or peptone-like substances are similarly treated, the precipitate produced by picric acid is not readily soluble in excess of reagent. The precipitate obtained in this way was called fraction I.

That part of the protone which was not precipitated in this way, after removal of the picric acid, was precipitated by the silver baryta method (which also precipitates protones). The precipitate was freed from silver and barium and the resulting solution treated with sodium picrate at neutral reaction. The precipitate provided fraction II. and the filtrate fraction III. Fraction I. was further subdivided into fractions Ia and Ib by purification of the part (Ib) by means of the copper sulphate compound.

Pringle estimated the arginine in clupeine to compare with the analyses of these fractions. His results expressed as percentages of the total nitrogen were :—

Clupeine, 88·7 ; 87·9 ; 89·1.

	Fractions.			
	Ia.	Ib.	II.	III.
Clupeone .	87·6	87·2	88·8	87·0

Goto obtained similar results.

These values were arrived at on the basis of calculation I. (p. 12). They show that there is no change in the ratio of arginine to monoamino-acids during the conversion of clupeine to clupeone. The ratio corresponds with the accepted formula a_2m which requires 88·9 per cent. of arginine. By employing calculation II. an arginine-nitrogen value 2 per cent. higher is given, but there is no appreciable departure from the formula a_2m.

The molecular weight determination by Goto mentioned above fits in with the hypothesis that the simple combination arginine + arginine + monoamino-acid is present in the clupeone molecule. It has already been mentioned that the clupeone molecule contains the four monoamino-acids, alanine, serine, proline, and an aminovaleric acid. The combination of each of these four units with two molecules of arginine with the loss of two molecules of water gives calculated molecular weights of 401 ; 417 ; 427 ; 429. Goto found 419 and 424. Some of the elementary analyses of clupeone by Goto (48) agree with this molecular weight while others are still unexplainable.

Relative Arrangement and Type of Linking of the Units.

If a peptide linking, according to Emil Fischer's scheme, be assumed in the molecule of a protamine of the salmine type, the following possibilities have to be considered :—

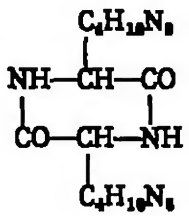
(I.) **aam-aam-aam . . .** or (II.) **ama-ama-ama . . .**

where a represents arginine and m monoamino-acids. In the formation of protones the linkages represented by the hyphens would be broken. Practical application of this assumption has presented difficulties. For example, Nelson-Gerhardt has shown that, on partial hydrolysis of salmine, monoamino-acids are found in combination with one another (133) ; Gross has confirmed the result with clupeine (54).

Gross has supported the assumption of a linkage between two arginine molecules by experiments suggested by the following consideration. Of the four nitrogen atoms of arginine, only the one attached to the α-carbon atom is estimated by formol titration or by van Slyke's method. If clupeine is completely hydrolysed and the reaction mixture is precipitated with phosphotungstic acid in the usual way, the precipitate should consist entirely of free arginine and a formol titration or estimation by van Slyke's method should give 25 per cent. of the total nitrogen as amino nitrogen. If, however,

the arginine in this precipitate is still partly in peptide combination, the number of reactive amino-groups will be reduced and values below 25 per cent. will be obtained. If the amino-groups are all in combination, the value will sink to zero.

Gross found values for the amino-nitrogen in the arginine fraction from the hydrolysate mostly less than 25 per cent. and he succeeded in obtaining by cautious hydrolysis a reaction mixture from which he was able to separate several fractions with different values for the reactive amino-nitrogen. In one case the amino-nitrogen estimated by van Slyke's method amounted to 3·3; 2·94; 2·46 per cent. of the total nitrogen. In another experiment only 0·8 per cent. was detected by a formol titration. It is probable that in these cases an arginine anhydride is present, i.e. a dioxypiperazine derivative of the formula—



If this assumption is correct, Gross' results show that two arginine groups in the clupeine molecule are joined by a peptide-like linkage. They do not indicate that a dioxypiperazine ring is actually preformed in the protamine molecule, for it is known that dipeptides are easily converted into their anhydrides.[1] Gross showed in a separate experiment that arginine in the form of the uncombined single molecules is not converted into the dioxypiperazine under the same conditions.

Kossel and Staudt's experiments on the prolonged action of 70 per cent. (by volume) sulphuric acid on clupeine led to similar results. From this reaction mixture a substance was isolated which on hydrolysis gave 100 per cent. arginine (using calculation II.). According to this all the amino-acids were split off from the protamine. The base obtained was not arginine, since a van Slyke determination indicated that not 25 but only 14·99 per cent. of the total nitrogen was present as free amino-nitrogen. After hydrolysis with sulphuric acid this value rose to 25 per cent. These results can only be explained by a linking of arginine molecules, such as is present in arginylarginine. Attempts at obtaining crystalline salts were unsuccessful.

Finally, one more phenomenon which arises from the acid hydrolysis

[1] See below, p. 53.

of protamines may be mentioned. This is the decrease in alkalinity, which was investigated by Goto (48) and Nelson-Gerhardt (133). It has also been observed in the hydrolysis of other proteins, and Sörensen (181) has traced it to a progressive change from the keto form —CO—NH— to the enol form —C(OH) = N—. Nelson-Gerhardt pointed out that serine is present among the hydrolysis products of this protamine. The rupture of an ester-like linkage of this hydroxy amino-acid would lead to the liberation of a carboxyl group and thus decrease the alkalinity. This reaction is illustrated by the following equation :—

$$\begin{matrix} COOH—CH—CH_2—O—CO—CHR \\ \quad\quad | \\ \quad\quad NH_2 \end{matrix} + H_2O = \begin{matrix} COOH—CH—CH_2—OH \\ \quad\quad | \\ \quad\quad NH_2 \end{matrix} + \begin{matrix} COOH—CHR \\ \quad\quad | \\ \quad\quad NH_2 \end{matrix}$$

Bergmann (9A) proposed the term " ester-peptide " for such a linkage.

Action of Alkalis on Protamines.

The action of alkalis differs from the action of hydrolytic ferments and acids mainly in the following points. Firstly, racemisation of the protamines occurs even at room temperature. Secondly, the guanidine group of arginine is destroyed with the formation of urea. There is also an evolution of ammonia, the origin of which is not known and which is possibly partly connected with this reaction. At the same time protone-like products are formed.

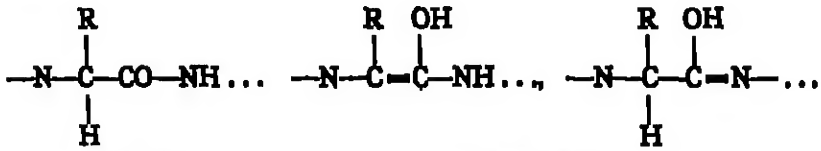
Kossel and Weiss (91, 92) observed in 1909 that, by the action of sodium or barium hydroxide at room temperature, or in an incubator, the optical activity of the protamines almost entirely disappeared. It cannot be assumed at once that this change is a racemisation. The rotation of so complex a molecule is in reality the resultant of several structural changes taking place within the molecule, which influence the rotation in different directions. If the molecule is partially disintegrated, some of the groups with lævo-rotation may disappear whilst those with dextro-rotation may remain unchanged. In such a case the lævo-rotation would diminish and the final result would be nearer a dextro-rotation (92).

The experiments of these authors led to the conclusion that such a change, if it occurs at all, cannot be regarded as the real cause of the inactivation, but that the inactivation of the whole protamine molecule went hand in hand with an inactivation of arginine, the unit predominating in quantity. If the inactivated protamine is subjected to acid hydrolysis, *dl*-arginine, and its decomposition product,

dl-ornithine, are formed, whereas acid hydrolysis of the original active protamine yields *d*-arginine. The action of sodium hydroxide must produce a structural change which renders the arginine optically inactive while it is still combined in the protein molecule. Subsequent racemisation of the arginine, or ornithine, is excluded under the given experimental conditions. It therefore appears that the units in the state of combination within the protein are more accessible to racemisation by alkalis than in the state of free amino-acids.

Kossel and Weiss extended these researches to the higher proteins with similar results (93). The optical inactivation occurs with gelatin and other proteins; on acid hydrolysis of racemised gelatin they obtained *dl*-histidine besides *dl*-arginine, while lysine was only partially racemised.

Dakin (17) gave a very simple and convincing explanation for this reaction. He pointed out that in amino-acids which are bound by peptide linkages in the protein molecule a change from the keto form to the tautomeric enol form is very probable as soon as they are subjected to the action of alkali. In this change the carbon atom of the amino-acid forms a double bond with one of its neighbouring atoms and thus loses its asymmetry. If now in any way, e.g. as a result of hydrolysis, the amino-acid reverts to the keto form a racemic substance results. The following scheme serves to illustrate this reaction :—

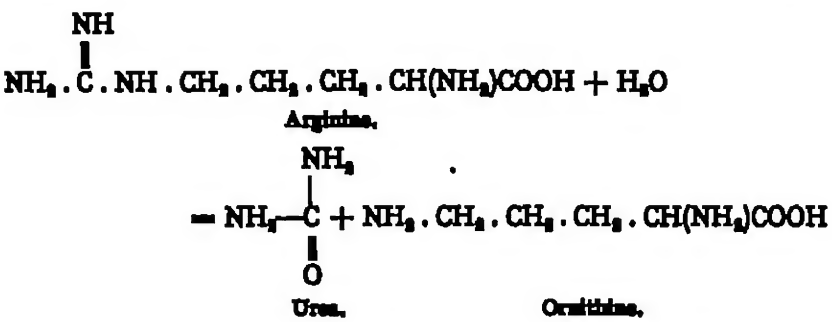


Keto Form. Two Enol Forms.

In the higher proteins (gelatin and casein) Dakin and Dudley (18) found that a series of other amino-acids also racemised under these conditions, but proline retained its optical activity. They emphasised the importance of these investigations, which arose from a study of the protamines, for the exploration of protein structure. It is to be expected from these observations that those amino-acids whose carboxyl group does not take part in the peptide linkage would not be concerned in the racemisation.

Dakin and Dudley also observed the *complete* resistance of the higher proteins racemised by alkali towards proteolytic ferments. This peculiarity is not explained. The phenomenon does not seem to have been yet investigated in the protamines.

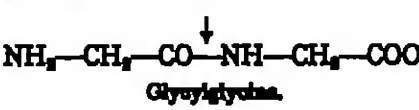
Besides racemisation Kossel and Weiss (92, 93) observed another reaction which occurred during the action of alkalis on the protein molecule. It consisted in the hydrolysis of combined arginine with the formation of urea. Protamine derivatives can be thus prepared which on decomposition yield ornithine instead of arginine. This reaction had already been described by Schulze and Likiernik (159) and Schulze and Winterstein (160, 161) soon after the discovery of arginine by Schulze; it led to the discovery of the constitution of arginine by Schulze and Winterstein. The reaction is expressed in the following equation which also represents the action of arginase on arginine :—



According to Kossel and Weiss this reaction occurs under the influence of alkali, if the carboxyl group of the arginine takes part in the peptide linkage, as is the case in the protamines.

Views on the Constitution of the Protamines.

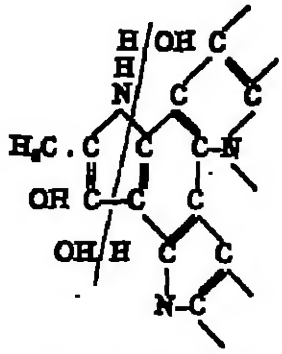
In the foregoing sections some observations were made on the nature of the linkage of the amino-acids in the protamine molecule. The contents of this section have thus, to some extent, been anticipated, but further possibilities exist for the structure of the protamines. The discussion on the hydrolysis products of clupeine and salmine was based upon the hypothesis that in the protamine molecule an amino-group of one unit is joined by loss of water to the carboxyl group of the adjacent unit. The peptide linking of two molecules of glycine giving glycylglycine is an example :—

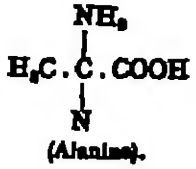


This compound is broken into its parts by adding a molecule of water at the point shown by the arrow. Experimental proof of this structure was given by Emil Fischer (40, 42). The protein molecule is thus conceived as an association of amino-acids, the atomic structures of which are already built up within the association. This structure explains the formation of amino-acids by hydrolysis whether it is affected by acids, or alkalis, or ferments. The formation of amino-acids by hydrolysis of proteins by ferments, a reaction which proceeds so easily and without violence at ordinary temperature, as well as the production of typical peptides, support this view. This conception is emphasised by the fact that artificially prepared peptides are hydrolysed by the same ferment, erepsin, which is required to liberate the amino-acids from the protein molecule. It is also known that the animal organism, in the formation of hippuric acid and glycocholic acid, performs reactions which are similar to those taking place in the formation of peptides.

This hypothesis by no means excludes other methods of linking, as Fischer has already pointed out (41). The great variety of the proteins, the difference of their functions and the occurrence of other reactive groups than COOH and NH_2 in the amino-acids (e.g. OH and SH) allows for other structures besides the peptide type of linking in the proteins.

Fischer's views are not entirely supported by the results of Troensegaard's exhaustive and careful researches. Troensegaard points out that, in the investigation of such a complicated organic structure as occurs in proteins, the exclusive use of hydrolysis as a method of examination is liable to lead to one-sided conclusions. He therefore conducted the cleavage in such a way as to avoid the use of aqueous solutions, and obtained a high yield of pyrrole like products when he subjected acetylated proteins to hydrogenation (186): "Neither aliphatic amines, nor aliphatic amino alcohols, which would be expected if proteins consist of a peptide chain of amino-acids were found in the reaction" (187). He concluded that hydroxypyrrole compounds were present in the proteins. The following provisional model explains the formation of amino-acids from pyrrole rings by hydrolysis, and how amino-acid formation is possible without peptide linkages:—

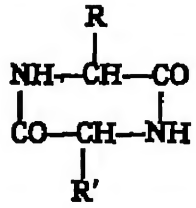




(Alanine).

In this case the amino-acid alanine is obtained by the rupture of an hydroxypyrrole ring by the addition of two molecules of water.

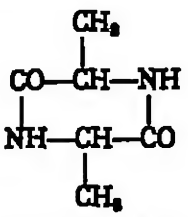
Attention has already been directed to another ring system which arises from the enzymatic hydrolysis of proteins. Ritthausen (148) and Sälaskin (154), and others, recorded the presence of a dioxypiperazine, leucine anhydride, a substance which was first described by Bopp in 1849. Dipeptides are formed by carboxyl-amino combination from two amino-acid molecules by the loss of one molecule of water; if a second molecule of water is lost, ring closure occurs and a dioxypiperazine is formed:—



An example has been given above (p. 48), but it was also mentioned that the pre-existence of such a ring in the protein molecule must not necessarily be inferred because it appears after hydrolysis of the proteins, since dipeptides are easily converted to dioxypiperazines by boiling with acid (Brigl (15), Abderhalden and Komm (3)). The work of Abderhalden and of Bergmann, however, has provided information which makes the existence of ring structures in the protein molecule probable. These researches are of outstanding general interest in protein chemistry, but have not as yet any special application to the protamines. The work of Bergmann applies essentially to the realm of synthetic structural chemistry, but it must be considered in regard to the occurrence of serine in the protamine molecule.

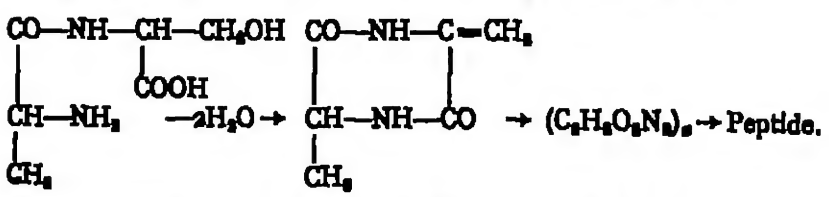
In a series of important papers Bergmann showed that certain

dioxypiperazines can be converted into substances which are very reactive, have a tendency to pass over into substances of high molecular weight, and also form peptides on hydrolysis. Of special interest is the preparation of a substance whose relationship with the dioxypiperazines is characterised by its conversion into alanine anhydride by catalytic hydrogenation, while on hydrolysis it yields a tetrapep-



Alanine Anhydride.

tide. Such a substance is formed by loss of water from the peptide combination of alanine and serine or of glycine and serine. A methylenedioxypiperazine, which has a tendency to change into a product of high molecular weight, is formed from the peptide. Either dioxypiperazine or peptide structures can then be split off according to the method of attack (10). The following is an example :—



Alanylserine. 3, Methylene-6-methyl-dioxypiperazine. High M.W. compound.

Bergmann and Stather (11) showed that if alanylcystine peptide is used in place of alanylserine a cystine containing dioxypiperazine is obtained whose behaviour in comparison with the peptides mentioned shows clearly that " ring closure exerts a profound influence on the affinity relations within the peptide molecule."

The Preformed Acidic and Basic Groups of the Protamines.

The theory briefly described in the foregoing section assumes that in the original protein molecule there are groups which first attain the form COOH or NH_2 during hydrolysis. The protein molecule must be assumed to contain preformed carboxyl and amino-groups since the proteins are ampholytes. The preformed carboxyl and

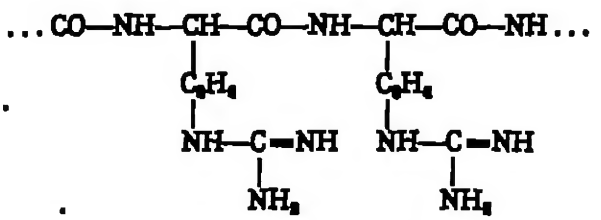
amino-groups determine the acidic or basic character of the protein. The protamines and histones are basic. Two problems need to be examined, first the method of the linkage within the molecule—whether it is of the chain or peptide type or whether it is the ring form; secondly the nature of the free groups not concerned in the linkages.

Goto's (48) alkalimetric experiments with clupeine are important. He found that the combining power of clupeine with acids was as great as that of the arginine contained in the protamine. One molecule of free arginine has one alkali equivalent, so that of the four nitrogen atoms of arginine one is responsible for the alkaline reaction in the titration.

$$NH_2C(NH)—NH—CH_2—CH_2—CH_2—CH(NH_2)—COOH.$$

Arginine.

Arginine has two free amino-groups. One is the α-amino-group of the ornithine portion and the other belongs to the guanidine nucleus. Which of the two is free and confers on the protamine molecule the alkalinity which can be determined quantitatively? The answer is given by the behaviour of the two groups towards nitrous acid. Under the conditions determined by van Slyke (116) the α-amino-group is decomposed by this reagent with the quantitative evolution of its nitrogen, while the amino-group of the guanidine residue (as also the NH group present in the peptide linkage) is not attacked. Kossel and Cameron (97) acted upon clupeine and salmine with nitrous acid and found that no nitrogen was evolved by the protamines. The α-amino-group therefore was combined in the molecule and the amino-group of the guanidine nucleus was responsible for the strong basic reaction of the molecule, and existed in the free state. On the basis of the peptide theory the following structure was indicated:—



The existence of a free amidine-group in the protamine molecule is supported by other observations.

The formol titration devised by Sörensen (180) supports this result.

If formaldehyde is allowed to react with a free amino-group the following reaction takes place :—

$$R — NH_2 + CH_2O \rightarrow R — N = CH_2 + H_2O.$$

The amino-group is eliminated and its basicity disappears. If an acid group is present of which the acidity before the action of formaldehyde was wholly or partly neutralised by the amino-group, its acidity is more readily recognised after removal of the amino-group, and it can then be determined by titration. A free amino-group belonging to the guanidine nucleus does not combine with formaldehyde (Sörensen). It can thus be ascertained in this way whether the basic nature is due to a guanidine group, or another amino-group, or both.

Kossel and Gawrilow (98) subjected a series of protamines to this test. Clupeine, salmine, coregonine, salvelino, and scombrine (examined in the form of the sulphates) showed no formol-titratable nitrogen. Their basicity cannot be due to the α-amino-groups of amino-acids bound in the protamine molecule and must therefore depend upon the guanidine group. A terminal α-amino-group cannot be present which must be the case if a peptide linkage is assumed. There is therefore a difficulty in following up the peptide theory.

Amongst the protamines of the salmine group cscoine sulphate was the only one to behave abnormally. In this case the nitrogen reacting with formaldehyde amounted to 1·9 per cent. of the total nitrogen. This result might be due to impurities or preliminary decomposition.

The behaviour of certain protamines which contain lysine units is very different. The presence of a reactive amino-group in these is shown by the action of nitrous acid as well as by formol titration.

Protamine.	Percent. of Total Nitrogen reactive towards	
	Nitrous Acid (Kossel & Contessini).	Formaldehyde (Kossel & Gawrilow).
Sturine sulphate	6·6	6·9
" "	6·9	6·3
" "	—	6·4
Cyprinine sulphate (mixture of two forms)	23·6	12·8
" " " " "	—	13·0
" " " " "	—	13·2
Crenilabrine sulphate	—	7·5

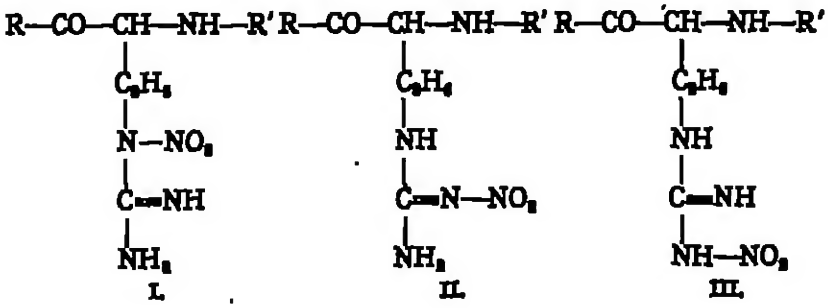
Cyprinine which contains more lysine than sturine or crenilabrine shows a higher content of reactive amino-nitrogen. A definite proportional ratio was not shown by the two sets of data.

Several of the higher proteins are lysine-free or nearly so, e.g. zein. They behave similarly towards nitrous acid. The lysine containing proteins form compounds with formaldehyde and can be titrated by formol; zein cannot. It is not yet certain whether the lysine bound in the higher proteins has one or two free amino-groups. It must not be assumed that the mode of linkage of lysine in proteins is the same in all of them. The values found for sturine give ground for the assumption of two reactive amino-groups on the lysine bound in the protein (Felix, 31).

These data are in good agreement with an earlier observation by Skraup and Hoernes (168). If casein is treated with nitrous acid, lysine is not present among the products of the subsequent hydrolysis. At least one amino-group of the lysine bound in the protein is therefore in the free state.

The manner of the entry of a nitro-group into the protamine molecule also fits in with the assumption of a free guanidine group. Kossel and Kennaway (115) prepared the nitro-derivative of clupeine by grinding clupeine sulphate in small portions with an ice-cold mixture of concentrated and fuming sulphuric acids and adding fuming nitric acid, keeping the mixture well cooled. On dropping the mass into ice-cold water, the nitro-product separated as a white precipitate. It is soluble in alkalis and reprecipitated from the solution by acids and can be purified in this way. It gives a biuret reaction and yields nitroarginine on hydrolysis. The latter can be obtained by the nitration of arginine and is probably a derivative of the asymmetric nitroguanidine prepared by Thiele (183). The guanidine group of clupeine thus behaves on nitration just like the guanidine group of arginine. Nitro-derivatives with similar properties have also been prepared from salmine (195) and sturine (96) and from all nitroarginine was obtained as a hydrolysis product. Similar nitro-derivatives can be obtained from the higher proteins (Kossel and Weiss, 99).

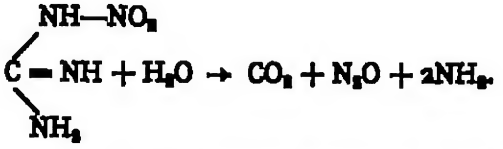
On the assumption that the peptide linking occurs through the α-amino-group the entry of the nitro-group into the arginine residue can be represented by the following formulæ :—



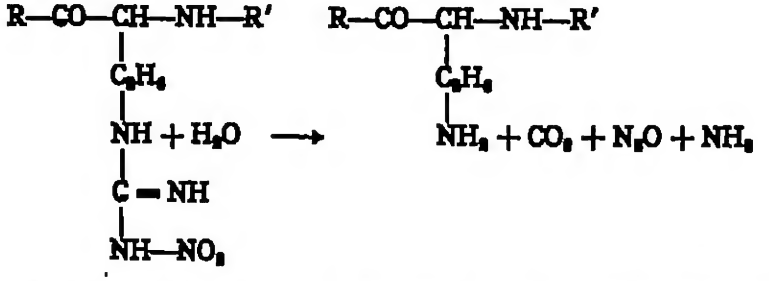
Formula I. can be rejected as ornithine can be formed from it (see below). From analogy with nitroguanidine formula III. is the most probable.

At present only two protein units besides arginine are known which take on a nitro-group by nitration of the whole molecule, namely tyrosine (Inouye, 70), and probably phenylalanine (Nenoki and Sieber, 134). Neither are present in clupeine.

These nitro-derivatives undergo a remarkable change under the influence of alkalis. The amidine-group of guanidine is decomposed with the formation of carbon dioxide, ammonia and nitrous oxide. Kossel and Weiss (99) proved that the decomposition of nitrated proteins occurred in a similar way to the decomposition of nitroguanidine observed by Thiele (183).



According to formula III. (above) the reaction can be expressed in the following way:—



Kossel and Weiss proved the identity of the evolved gas with nitrous oxide. The amount of nitrous oxide corresponded to the nitration of about 90 per cent. of the arginine present in the clupeine. In this way the arginine bound in the protein is deprived of its amidine-group, while the ornithine portion remains in combination in the protein molecule. This was proved by Kossel and Weiss by isolating from the reaction mixture a protone-like substance which on hydrolysis with boiling sulphuric acid yielded ornithine instead of arginine. The reaction proceeds in the same way if ammonia instead of sodium hydroxide acts upon the nitrated clupeine.

Before these researches were commenced, it was known that the amidine-group of guanidine could be removed from combination with

the protein molecule without destroying the cohesion of the whole. Kossel and Dakin (84) examined a reaction product which had been obtained from clupeine sulphate by acting upon it with an extract of the mucous membrane of the small intestine for many months. It was a protone-like substance and it was separated from the other reaction products and from free ornithine. If this "β-clupeone" was subjected to acid hydrolysis a considerable amount of ornithine as well as arginine could be detected among the products, whereas under the same conditions no ornithine was formed from clupeine. The cause of the reaction was not at first clear. Kossel and Dakin thought it might be explained by the action of an enzyme similar to arginase, but the researches of Kossel and Weiss (59, 60) showed that alkali acted in the same way.

The experiment was performed in the following way: Clupeine sulphate was dissolved in N/2 baryta and digested at 40° for 20 days. This reduced the rotatory power to a small value. A substance which behaved like clupeone was precipitated from the reaction mixture by the silver baryta method (p. 5). Free ornithine could not be present in this precipitate since it is not precipitated in this way, but still *dl*-ornithine was formed from this precipitate on acid hydrolysis. This result led to the conclusion that a part of the arginine bound in the protein is converted to ornithine whilst still combined in the protein by the treatment with alkali.

All these facts favour the assumption that in the protamines so far examined there is a free guanidine group not taking part in the peptide linking. They are also of interest from the physiological point of view since they show that a urea-forming group is loosely bound in the protein molecule and that the animal organism need not break up completely the structure of the protein molecule for the formation of urea. The urea can be taken from the protein molecule without breaking the peptide linking.

Sakaguchi (153) has recently studied this reaction and found that the decomposition of the arginine bound in the protein can also be brought about by alkali in the presence of sodium hypochlorite and hypobromite.

This discussion raises a similar question with regard to lysine whether one or both of its amino-groups are free and not concerned in the intramolecular linking. About this there is a difference of opinion chiefly owing to the varied nature of the structures of the proteins examined (166A, 31). Among the protamines sturine has received the most attention, and in this case analyses support the assumption of two free amino-groups in lysine.

Another method of estimating the reactive nitrogen-containing groups in the protein molecule is by the determination of the alkyl groups which can be introduced. Skraup and Krause (163) and Skraup and Böttcher (164), as in the researches on the action of nitrous acid mentioned above, started with the idea "of making chemical changes in the protein and then determining by hydrolysis upon which groups such changes had occurred." The higher proteins on methylation were found to undergo such a change that tyrosine, lysine, histidine, and arginine as such were entirely or partly absent from the products of hydrolysis. Rogozynski obtained similar results with clupeine (150).

Herzig and Landsteiner (62) examined the action of diazomethane on various proteins, and estimated quantitatively the methyl groups taken up by nitrogen. They found a higher value for N-methyl in the lysine-rich sturine than in the higher proteins.

Edlbacher (25, 26, 27) systematically estimated the methyl groups taken up by the nitrogen on methylation with dimethyl sulphate and compared N-linking of methyl groups with reactivity with formaldehyde. The essential part of these investigations is summarised in the following table :—

Protein.	Formol N. Percent. of Total N.	N-Methyl Number.	Lysine Content.
Gelatin	3·4	15·0	+
Casein	5·5	16·0	+
Edestin	3·3	15·0	+
Gliadin	—	—	—
Zein	—	—	—
Thymushistone	19·5	22·6	+
Gadushistone	16·1	40·5	+
Cyprinine (mixture of two forms)	18·7	62·9	+
Sturine	8·6	24·0	+
Clupeine	—	24·0	—
Salmine	—	9·0	—
Rascine	—	—	—
Scombrine	—	—	—

"Formol N" represents the number of nitrogen atoms which combine with formaldehyde by Sörensen's method. The "N-Methyl" number is the number of methyl groups per 100 nitrogen atoms which combine with nitrogen on exhaustive treatment with dimethyl sulphate in alkaline solution. These numbers do not show the distribution of methyl groups on individual nitrogen atoms.

Edlbacher's results show that in most cases the proteins which react with formaldehyde and with nitrous acid are capable of taking

on methyl groups under the conditions employed. Pro
lysine take on methyl groups, cyprinine, the richest i
on the most. Lysine-free proteins with certain excep
and salmine) are not methylated under these condi
exceptions are remarkable since the differences cannot
by other methods of investigation.

On hydrolysis proteins form new amino (and carboxyl) g
The methods of Sörensen, van Slyke, and Edlbacher would be expe
to give higher values with albumoses, peptones, and peptides than
with the original proteins. This is, in fact, the case. Formol titra-
tion, and also the titration method of Willstätter and Waldschmidt-
Leitz are convenient and useful methods for following the course of
protein hydrolyses.

If protamines are treated with acid chlorides in the presence of
alkali, the reaction is not confined to the free amino-groups. The work
of Hirayama (64) mentioned below (p. 63) proves that the imino-
nitrogen of iminazole combines with the naphthalenesulphonic group.
Benzenesulphonic chloride and β-naphthalenesulphonic chloride were
used by Hirayama, since the extent of their combination could be
easily calculated from the sulphur content of the product of the
reaction. The number of acyl groups taken on per 100 nitrogen atoms
were :—

	Clupeine.	Sturine.
By the action of naphthalenesulphonic chloride .	14-15	18·3
„ „ benzenesulphonic chloride . .	25	22·4-24

This work was continued by Edlbacher and Fuchs (29) and ex-
tended to the higher proteins. They found the following number of
naphthalenesulphonic groups for each 100 atoms of nitrogen :—

In clupeine	16·1
In salmine	18·0
In sturine	16·0

The higher typical proteins gave lower values :—

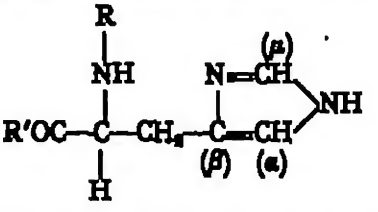
Gelatin	13·5
Casein	11·7
Edestin	9·2

The strongly basic histone gave a value 15·1, which is close to the
protamine values. All these differences appear trifling compared to
the big fluctuations in formol and N-methyl values. But the numbers
increase on hydrolysis. Thus Hirayama showed that for every 100

nitrogen atoms of clupeone there were 43 naphthalenesulphonic groups, or for every 9 nitrogen atoms 3·9 hydrogen atoms can be substituted by acyl.[1]

The same question regarding the position of arginine in the protamine molecule applies equally to histidine,[2] i.e. whether the iminazole group of the histidine-containing protamines (sturine and percine) exists in the free state and takes no part in the peptide linking. Comparison of the basicity of the whole protamine molecule with the relative proportions of the basic units (a method which has yielded clear results in the salmine group) gave no reliable evidence with sturine, as the basicity of the iminazole group is very feeble and it is still doubtful how many free amino-groups can be ascribed to lysine. The lysine-free percine has not yet been examined along these lines.

The behaviour of the histidine combined in the protamine molecule must be examined. If both the carboxyl and amino-groups of histidine take part in the peptide linking (R, R′) as with the other amino-acids of the protein molecule, the structural formula for the histidine contained in the protamine is as follows :—



Two series of reactions worked out by Pauly can indicate the position of the iminazole nucleus in the protamine molecule. One reaction is the coupling with diazo compounds and the other is the combining power with iodine.

Pauly's experiments (137, 140) on the diazo-coupling of histidine which is based upon Wallach's observation of the formation of a coloured substance, show that it is dependent upon the imino-group of iminazole being free. If the hydrogen atom of this imino-group is replaced by an acyl group, the diazo-coupling does not occur.

Pauly proved that the histidine combined in the sturine molecule coupled with diazo-compounds in the same way as free histidine.

[1] On the relation of these figures to the hypothetical formula of clupeone, see Hirayama, *Zeit. physiol. Chem.*, 55, 288 (1908).

[2] Pauly (136) first showed that histidine contained an iminazole ring combined with an alanine residue, and his work was confirmed by Knoop and Windaus (734) and Knoop (732).

Later Hirayama (64) found that this coupling [1] did not take place if benzenesulphonic or naphthalenesulphonic groups had been introduced into the sturine previously, thus depriving the iminazole imino-group of its hydrogen. Pauly concluded from his experiments that the imino-group of iminazole, i.e. that group which could form a peptide linkage, was present in the original sturine in the free reactive state. This conclusion was confirmed by Hirayama's work. Gerngross (47A) showed that a histidine ester acylated on the imino-group of the iminazole nucleus was fairly easily decomposed. A peptide linkage at this point, therefore, probably does not form.

Pauly (138, 139) also examined the iodo-derivatives of iminazole and histidine and estimated the absorption of iodine on iodinating the iminazole groups present in histidine acylated on the nitrogen in the side chain (benzoyl-histidine and nitrobenzoylhistidine) and in histidine anhydride. Comparison of the results with the values for the absorption of iodine by sturine showed that they were as large as those for the histidine derivatives in which the iminazole group is left free.

[1] Pauly (137, 140) believes that the imino-group of the iminazole is the first point of attack of the colour-forming group, but that linking with carbon follows with the formation of true azocompounds.

PART II.

THE HISTONES.

CHAPTER I.

CHARACTERISTICS OF THE HISTONE GROUP.

The histones differ from the protamines in having greater variety in the units. In this respect they are more like the typical proteins. They form a group of substances containing members of different types. The membership of a group must be decided on the basis of knowledge of chemical structure. The limits of such a group are always arbitrary; for it must be assumed that there are transition forms resembling protamines and transition forms resembling the complex proteins, in which basic groups do not control the nature of the molecule, and it is a matter of choice whether or not such transition forms are classed as histones.

The most definite characteristic of the histones is their basic nature which is due to a preponderance of free amino-groups. The high *arginine content* of the histones makes it possible to assume, from analogy with the protamines, that it is probable that the basic properties are in general connected with free guanidine groups. On the other hand, the existence of higher proteins *rich in histidine* (e.g. globin) and also the analogy with certain protamines raises the question whether in addition to free guanidine groups the free imino-group of the iminazole nucleus does not behave in a similar way to the amino-group of guanidine and whether globin should not also be considered as a histone. The separation is purely arbitrary.

Owing to the imperfect insight into the internal structure of the protein molecule external properties have to be relied upon for placing a protein in the histone group. One would base the classification upon one or more reactions by which the histones could be distinguished from the other proteins, but such a reaction is not known. The histones are characterised by the concurrence of *several* properties and reactions, which, however, are not always all present.

These reactions and characteristics are given below. If too strictly applied, almost every one can lead to false conclusions, since either they are occasionally given by other proteins or they fail to work with some histones.

(1) According to present ideas, a protein is regarded as a histone from the multiplicity of its units like a higher protein and at the same time from its basic character. The basic character finds expression in the position of the isoelectric point in the alkaline range and in the capacity for combination with acids. The latter can be quantitatively determined by analysis of the salts and by electrometric titration. The isoelectric point and electrometric titration methods have at present only been applied to thymus histone.

(2) So far as our present knowledge goes, a high arginine content must be considered as a general characteristic of the histones. The arginine nitrogen content amounts to 19 to 30 per cent. of the total nitrogen.

(3) The histones form sparingly soluble compounds with non-histone-like proteins which behave similarly to the protamine compounds already described.

(4) With an excess of ammonia a histone solution gives a precipitate which is rapidly transformed on standing with the precipitant into an insoluble modification of the histone. Certain histones do not give this reaction.

(5) The histones are precipitated by alkaloidal precipitants in *neutral solution*.

(6) By the action of pepsin-HCl "histopeptone," a basic peptone-like breakdown product, is formed.

(7) The histones have so far always been found in combination with nucleic acids, often accompanied by other proteins. Compounds of nucleic acids with proteins are generally called nucleoproteins. The nucleic acids can combine with the typical non-basic proteins as well as with the strongly basic protamines and histones. In the latter cases the salt-like character of the compound is especially definite. This is shown by the fact that the base can be dissolved out from the compound with nucleic acid by a strong acid, since the nucleic acids are displaced by strong acids. An example of this is provided by the sperm heads of various animals. In certain fish (e.g. Gadidæ), compounds of nucleic acids with histones are present, but in mammals the nucleic acids are combined with other types of proteins which possess no marked basic properties. In the first case the proteins (histones) are dissolved out by hydrochloric acid, in the latter they are not. By the prolonged

action of sodium chloride the compounds of the nucleic acids with the histones and protamines are so altered that the basic proteins cannot be extracted with acids. These changes can be partially reversed by washing out the sodium chlorides (Banus).

The grouping of the histones with the protamines is the result not only of their chemical properties as basic proteins, but also of their common relation to the chief constituents of the cell. This is discussed in more detail below. The combination of the biological and the chemical considerations gives an insight into the nature of this biochemical group.

The Individual Histones.—In the chemical composition of the histones there are some structural relations, and hence some properties, by which they can be distinguished from the other proteins, while others are common to both. The latter will not be discussed except incidentally in the following chapters since a consideration of them in detail would mean an extensive description of protein chemistry which does not come within the scope of this monograph. For the same reason the analytical methods which aim at elucidation of the histone molecule will not be dealt with. These methods are not different to those usually applied for the typical proteins. Finally it can be stated briefly that the histones contain besides the bases, arginine, histidine, and lysine, the monoamino-acids of the usual proteins, that ammonia is formed—in more or less large quantities—on acid hydrolysis, and that they give essentially the same precipitation reactions as the typical proteins and display colloidal character in solution.

CHAPTER II.

THE HISTONE OF THE ERYTHROCYTE NUCLEUS.

This substance, the discovery of which in 1884 led Kossel (74) to formulate the group, was obtained from the red corpuscles of goose blood. It is present as a salt-like compound with nucleic acid which is insoluble in water, but which has not been further examined. If these blood corpuscles, isolated in the ordinary way, are treated with water and the insoluble mass of nuclei remaining is placed in dilute hydrochloric acid (after it has been washed with water to decolorise it), all the histone goes into solution as hydrochloride. It can be salted out from the solution by sodium chloride and then freed from salt by dialysis when it passes into solution again. If this solution is concentrated at low temperature and precipitated by alcohol with the addition of ether, a water-soluble preparation of the histone hydrochloride is obtained. If the watery solution is treated with excess of ammonia, the histone is converted into an insoluble modification.

The neutral solution of this histone salt is precipitated by more or less complete saturation with ammonium sulphate, ammonium chloride, magnesium sulphate, sodium chloride or sodium carbonate. The solution is precipitated by lime water and sodium hydroxide besides ammonia, but the precipitate is readily soluble in excess of sodium hydroxide. Nitric acid produces a precipitate which dissolves on warming and comes down again on cooling. No coagulation occurs on boiling the aqueous solution. The aqueous solution gives a biuret and Millon reaction.[1] The precipitate brought down by ammonia contains 52·31 per cent. C; 7·09 per cent. H; 18·46 per cent. N; 0·65 per cent. ash.

From Ackermann's investigations (4) it appears that the histone in the erythrocytes of bird's blood occurs in salt-like combination

[1] The lead sulphide and the tryptophan reactions were given very feebly, possibly owing to contamination with other protein substances.

with nucleic acid. Ackermann used the nuclear substance of the erythrocytes of hen's blood, isolated by Plenge's method and extracted with alcohol and ether, for a determination of the nucleic acid by estimating the phosphorus. He found that 100 grams of the dried nuclear substance contained 42·16 grams nucleic acid. From the total nitrogen of the nuclear substance the nitrogen left after deduction of the histone nitrogen corresponded with the assumption that 100 grams nuclear material contained 42·16 grams nucleic acid and 57·82 grams histone, and that no appreciable amount of any other nitrogen-containing substance was present.

CHAPTER III.

THE HISTONE OF THE THYMUS GLAND OF THE C

This histone is present in the cells of the thymus gland i: tion with nucleic acid, but this compound is, in distinct of the erythrocyte histone, soluble in water. Lilienfeld (118, prepared from an aqueous extract of the thymus gland a su which contained the histone in salt-like combination with nucle It is known as "nucleohistone."

A. The Nucleohistone of the Thymus Gland of the Calf.

According to Lilienfeld the aqueous extract of the lymph cells or of the whole gland is treated with acetic acid. The precipitate is dissolved in water which has been made faintly alkaline with sodium carbonate and again precipitated with acetic acid. The precipitate is again purified in this way and then dehydrated with alcohol and ether. The nucleohistone is thus obtained as a white powder.

The substance prepared by Lilienfeld proved to be insoluble in water but soluble in solutions of several neutral salts and of sodium carbonate, sodium hydroxide, and ammonia. From these solutions it was precipitated by acetic acid and by alcohol. According to Gamgee and Jones (45) the faintly alkaline solution is dextro-rotatory. By the action of dilute hydrochloric acid it is decomposed with the formation of the histone. A protein and nucleic acid containing residue, which Lilienfeld called "leuconuclein," is also formed and from this a nucleic acid with a phosphorus content of 9·9 per cent. is obtained by more drastic action.

Lilienfeld's statements were confirmed and supplemented by various authors, first by Malengreau (122) and Bang (7, 8) and then by the fundamental work of Hulskamp (66, 67, 68). The following are the most important results:—

(1) The nucleohistone shows acidic and basic properties (the latter due mainly to the histone portion). The acidic groups predominate so that the whole nucleohistone behaves as an acid.

(2) This is shown by the fact that in neutral or feebly acid solution it moves to the anode as a negatively charged ion. It is deposited at the anode from the solution of its sodium salt. This deposition occurs with the sodium-free " acid " of the nucleohistone which gives the same qualitative reactions. The calcium salt obtained from this electrolytically obtained product gives, on analysis, the phosphorus and nitrogen values of the calcium salt of nucleohistone (Hulskamp).

(3) The sodium salt of the nucleohistone thus appears ionised in solution. The same applies to the other soluble salts of the nucleohistone. As an acid the nucleohistone can take up still more added histone. This happens if the sodium salt of the nucleohistone is mixed with nucleohistone hydrochloride when a histone-richer substance is produced with the formation of sodium chloride (Hulskamp).

	Nucleic Acid.	Histone.
Original nucleohistone . . .	60 per cent.	40 per cent.
Histone-rich substance . .	38 "	62 "

(4) If the electrolytic dissociation of the dissolved nucleohistone salts is depressed by the addition of equally ionised electrolytes, their solubility is diminished. Upon this depends the formation of the precipitate which occurs in a solution of the sodium salt of the nucleohistone if sodium chloride is added until its content reaches 0·6 per cent. to 0·9 per cent. More sodium chloride redissolves the precipitate (Hulskamp and Bang).

(5) Under certain conditions the nucleohistone forms insoluble salts with the alkaline earth metals. Such a salt is formed by double decomposition if a solution of the sodium salt of the nucleohistone is treated with sufficient calcium chloride to produce a concentration of 0·1 to 0·5 per cent. Since the aqueous extract of the thymus gland contains the sodium salt of the nucleohistone, this property is often utilised for the preparation of the nucleohistone.

(6) The nucleohistone prepared by Lilienfeld was found to be still impure. It was mixed with a phosphorus-containing substance which contained instead of the histone another protein richer in carbon and poorer in nitrogen. In the papers mentioned this substance was generally referred to as " nucleoprotein."

It is called " nucleoprotein X " since, as mentioned above, the term nucleoprotein refers to all compounds of nucleic acid with proteins and thus also includes the nucleohistones. The nucleoprotein X can be separated by fractional precipitation with ammonium sulphate (by which it is precipitated by a smaller concentration of the salt) and also on the basis of the greater solu-

bility of its calcium salt. This nucleoprotein does not show the characteristic precipitability by 0·9 per cent. sodium chloride mentioned above for the nucleohistone.

(7) According to Hulskamp the substance when freed from this nucleoprotein still cannot be regarded as homogeneous. It can be separated into two nucleohistones (α- and β-nucleohistones), one of which, with a phosphorus content of 4·4 to 4·5 per cent., is more readily precipitated than the other by 0·6 to 0·9 per cent. sodium chloride. The other contains 3·04 per cent. phosphorus. Both are rich in histone. On the basis of a communication by Bang on a nucleohistone preparation, Hulskamp considered the possibility whether the α-nucleohistone or even both the nucleohistones should not be still regarded as mixtures of substances with different high phosphorus contents.

Even if the nucleohistone has not yet been established as a chemical individual, the following analyses of this substance are of value for a general characterisation :—

	Lilienfeld, 1898.	Steudel,[1] 1913.	Hulskamp, 1922. (Ash-free Substance.)
C . .	48·46	48·38	48·79
H . .	7·00	6·92	7·03
N . .	16·86	16·81	16·37
P . .	3·08	3·11	—
S . .	0·70	0·72	—

Hulskamp found the following values for the calcium salt of the nucleohistone :—

	Prepared Directly (95).	Prepared from Nucleohistone Deposited Electrolytically at the Anode (97).
C . .	45·31	—
H . .	6·50	—
N . .	17·07	17·21
P . .	3·75	3·74
S . .	0·509	—
Ca . .	1·356	—

The preparation analysed by Hulskamp was free from the nitrogen poor "nucleoprotein X," but consisted of a mixture of the α- and

[1] The substance analysed by Steudel was prepared by following closely the directions of Lilienfeld. Thus there naturally remains the question whether or not the precipitate is a homogeneous chemical individual (175).

β-nucleohistones. For the preparation of the calcium salt of the nucleohistone, Hulskamp precipitated the thymus extract by 0·1 per cent. calcium chloride. The precipitate was dissolved in water with the addition of a few drops of dilute ammonia and reprecipitated, after filtration, with 0·1 per cent. calcium chloride. The precipitate is washed thrice with alcohol and then with ether and dried at 110° for analysis. In this way the calcium salt of the nucleohistone is obtained. It gives up its calcium to acetic acid.

By repeated precipitation with calcium chloride the impurity of nucleoprotein X is removed. Bang attained the same result by precipitation with 0·6 to 0·9 per cent. sodium chloride.[1] Steudel (176) observed that ether-soluble substances, which caused a cloudiness, are present in the aqueous extract of the thymus gland. It is necessary to remove these substances by extracting with ether.

As already mentioned above, the salt-like character is especially marked in those nucleoproteins which contain basic proteins. The compound of nucleic acid with protamine was previously called "protamine nucleic acid" (Miescher, Schmiedberg) and nucleohistone "histone nucleic acid" (Bang). Various reasons were advanced for these ideas, first by Bang (8) and later by Steudel (175). Steudel was the first to prove that the phosphorus of the nucleohistone was present exclusively in the nucleic acid component (thymus nucleic acid) and in no other form. He also found fairly good agreement on comparing the artificially prepared histone nucleic acid and the nucleohistone (176). He showed that the whole amount of histone could not be extracted by short treatment with hydrochloric acid from the artificially prepared histone nucleic acid nor from the nucleohistone. Only 16·5 per cent. of the histone went into the hydrochloric acid extract from the artificially prepared histone nucleic acid instead of 41 per cent. expected from the nucleic acid content. Steudel therefore assumed that by treatment of the nucleohistone with 0·8 per cent. hydrochloric acid (three times, each for half an hour) a less soluble acid salt of nucleic acid with the histone was formed from the original neutral salt and only the small amount of histone thus liberated goes into solution. The acid salt can behave as an anion and combine with other bases, e.g. calcium or sodium. Hulskamp (67) obtained complete separation into 40 parts histone and 60 parts nucleic acid by 14 hours' treatment with 0·8 per cent hydrochloric acid.

The work so far done on the nucleohistone points to the conclusion

[1] The more recent communications of Feulgen refer to the nucleo-histone as starting material for the preparation of the histone.

that the nucleohistone prepared from the aqueous extract of the thymus gland by different workers is really histone nucleic acid which is sometimes contaminated with nucleic acid compounds of other proteins. It is still unknown whether a compound of nucleic acid and histone is preformed in the thymus gland.

B. The Histone of the Thymus Gland.

(a) *Preparation of the Histone.*

As mentioned above the histone can be obtained ac Lilienfeld (p. 69) from the nucleohistone, its precursor in t extracts of the thymus gland, if the nucleohistone is extr dilute hydrochloric or sulphuric acid, or if a solution of the s of the nucleohistone is treated with dilute sulphuric acid these conditions the nucleic acid separates and can be removed by fi ing off or centrifuging. A good method for preparing the histone fr thymus tissue is given by Felix and Harteneck (56).

The thymus gland after removal of adhering tissue is minced. The pulp is poured into a wide-mouthed bottle with 2½ times its weight of distilled water and shaken for an hour to ensure thorough mixing. It is allowed to stand overnight in the ice chest. The almost uniform gelatinous mass is strained through two layers of muslin or a kitchen sieve. The nucleohistone is precipitated from the filtrate by adding several cubic centimetres of dilute acetic acid; for a quantity of 800 grams, the quantity to which the following figures refer, 10 to 15 c.c. are required. Sufficient has been added when the rose-grey colour changes to pearl grey. It is filtered through several thicknesses of paper. The filtrate should be quite clear and only coloured slightly yellow; it contains a basic peptone. The liquid is tested with acetic acid to make sure that the nucleohistone has been precipitated completely.

The residue on the filter is twice extracted with 500 c.c. water by being shaken with it for an hour. Except that less acetic acid is required for precipitation of the nucleohistone the treatment is the same as in the first extraction.

The nucleohistone is shaken up into a pulp with a little water and thinned by the further addition of from 1 to 1½ l. water. Dilute sodium hydroxide (about 5 c.c.) is added to give a neutral or feebly alkaline reaction when the colour changes back to rose-grey and the nucleohistone partly dissolves. After shaking for another hour 10 c.c. concentrated sulphuric acid (previously diluted with an equal volume of water) are added per litre of liquid to bring about the separation of the nucleic acid from the histone. The precipitate of nucleic acid is collected on a pleated paper. The filtrate is slightly opalescent. If it is clear, it contains hardly any histone; too little sulphuric acid has been added. For precipitation of nucleic acid the reaction must be definitely acid to congo red. The residue on the filter is shaken twice more with 500 c.c.

water containing 5 c.c. concentrated sulphuric acid. The histone is precipitated from the combined filtrates by adding three times the volume of 96 per cent. alcohol and allowed to settle. The supernatant liquid is siphoned off and the residue collected by filtration or centrifuging. After the alcohol has drained away, the histone is dissolved in warm water, the solution is filtered quickly and again precipitated. If one waits for a long time the histone is difficult to dissolve and cannot be filtered easily. The precipitation is carried out thrice. After the last precipitation the histone is dried with alcohol and ether. A white dusty powder is obtained. The yield amounts to 25 to 30 grams histone sulphate from 1 kg. gland.

From this preparation the histone can be obtained as the free base by precipitating the solution of the histone sulphate with ammonia. In this way the "denatured" insoluble histone is obtained. This change can be avoided by using very little ammonia followed immediately by the addition of alcohol (Felix and Harteneck). The histone then retains its solubility in water. After two more precipitations from water and alcohol it is free from sulphuric acid and ammonia.

There is another method, also due to Felix and Hartenack, for obtaining a soluble preparation of the free base. The histone sulphate is dissolved in water and made up to a known volume, an aliquot part is brought to the p_H of the isoelectric point (p_H 8·51) by addition of sodium hydroxide using cresol red as indicator. The calculated amount of sodium hydroxide is added to the main bulk of the solution and the resulting cloudy solution precipitated with an equal volume of 85 per cent. alcohol. The precipitate can be filtered off easily. The process is repeated twice more and the histone then dried with alcohol and ether. The white powder thus obtained only contains traces of sulphate, probably in the form of sodium sulphate.

(b) *Composition of the Thymus Histone.*

The following values have been found by elementary analysis of the histone:—

	Lilienfeld (1898).	Plimmer (1911).	Hedenius (1917).		Bang (1911).	Felix and Hartenack (1926).
			Precip. by $NaOH$.	Precip. Electrolytically.		Precip. at Isoelectric Point.
C .	52·34	52·37	—	—	—	—
H .	7·31	7·70	—	—	—	—
N .	—	18·35	18·14	17·98	18·05	17·46
„ .	—	—	18·10	—	18·35	—
„ .	—	—	18·03	—	—	—
S .	—	0·62	—	—	0·61	—

These values agree with those given above for erythrocyte histone.

The amounts of the basic hydrolysis products are given below as percentages of the total nitrogen :—

	Kossel and Kutscher, 1900 (64).	Felix and Hartenseck, 1907 (37).
Histidine . .	1·79	5·8
Arginine . .	25·17	27·1
Lysine . .	8·04	(9·7)[1]
Ammonia . .	7·46	3·2

The variation in the histidine values are in all probability due not to a difference in the composition of the preparations but to improvements in technique.

Kutscher (114) detected 6·31 per cent. tryosine and 3·66 per cent. glutamic acid amongst the hydrolysis products.

Abderhalden and Rona (2) also isolated the following amino-acids. With the exception of tyrosine the following values are to be regarded as the minimal values :—

	Per Cent.
Glycine	0·5
Alanine	3·46
Leucine	11·80
Proline	1·46
Phenylalanine	2·20
Tyrosine	5·20
Glutamic acid	0·53

(c) *Reactions and General Properties of Thymus Histone.*

Felix and Harteneck (37) obtained by their method a preparation of the histone which, after precipitation eight times as sulphate and four times with ammonia and alcohol, appeared under the microscope to be mainly crystalline. The free histone is only very slightly soluble in water but the ***hydrochloride*** and ***sulphate*** are readily soluble. The former in the presence of a small excess of hydrochloric acid dissolves in 70 per cent. alcohol and can be precipitated from the solution by ether. The sulphate is insoluble in 70 per cent. alcohol. According to Bang, the phosphate is sparingly soluble, and the nucleate almost insoluble. On addition of sodium picrate to a solution of the hydrochloride or sulphate, the picrate separates as a sticky mass. Salts of the histone are precipitated from neutral solution by the alkaloidal reagents (e.g. phosphotungstic acid, phosphomolybdic acid, and

[1] Calculated by difference.

potassium ferrocyanide). The thymus histone is salted out by ammonium sulphate, sodium chloride, and other soluble salts.[1] With nitric acid the solution gives a precipitate which is dissolved on warming and comes down again on cooling. It has already been stated that the thymus histone is precipitated by ammonia. The precipitation is influenced by the presence of ammonium chloride. According to Huiskamp the precipitation with ammonia is incomplete or does not take place, if chloride is present. Precipitation is often, therefore, made possible by previous dialysis. On the other hand, Bang (6) stated that the ammonia precipitation was favoured by the presence of ammonium chloride, and that the histone was soluble in excess of ammonia but was precipitated from this solution by ammonium salts. Like the protamines, the histones give a precipitate with proteins in neutral solution, but, according to Huiskamp, only with those proteins which are acid in character and therefore not with globin.

(d) *Electrolysis of the Salts of the Histons.*

The salts of the histone are dissociated in water. This is shown on dialysis. If a solution of the histone hydrochloride is dialysed for 24 hours in running water, not only has any possible excess of hydrochloric acid disappeared but also the solution has attained a definitely alkaline reaction to litmus but not to phenolphthalein. But the hydrolytic dissociation is not so complete as to remove all the hydrochloric acid. This is only possible if the histone is precipitated by sodium hydroxide. If the free base, which reacts alkaline to phenolphthalein, is dissolved in excess of sulphuric acid and dialysed, a sulphate is obtained which is also alkaline to litmus, and from which the sulphuric acid can be precipitated by barium chloride. The SO_4 group is thus present as an ion. In this way Huiskamp (67) showed that the histone salts were electrolytically dissociated. This was confirmed by his later work.

He electrolysed the aqueous solution of the histone hydrochloride, which had been dialysed and reacted alkaline to litmus, and showed that the histone was deposited at the cathode while the reaction at the anode was acid. The histone deposited is soluble in hydrochloric acid and, provided that the electrolysis has not lasted longer than 16 hours, qualitative and quantitative examination revealed no change (see nitrogen estimation, p. 74).

[1] Malengreau (122) claimed to have found that there were two histones present in the thymus gland which were distinguished by their precipitability on salting out with ammonium sulphate. Bang (8) proved this claim to be false.

(e) *The Isoelectric Point of the Thymus Histone.*

The isoelectric point of the thymus histone lies in the alkaline region at p_H 8·51 in contrast to most proteins which are known to possess acidic character.

Felix and Harteneck (96) determined the isoelectric point by mixing phosphate buffers of various hydrogen ion concentrations with a 1·5 per cent. solution of pure histone and measuring the change in p_H. As the following table shows, the addition of histone caused the least change in p_H at p_H 8·51. For these determinations 4·5 c.c. of 0·066 M buffer solution were mixed with 2 c.c. histone solution.

p_H of Buffer.	p_H of Buffer—Histone Mixture.	Difference.
7·97	8·38	+ 0·41
8·27	8·51	+ 0·24
8·46	8·54	+ 0·08
8·51	8·52	+ 0·01
8·66	8·59	— 0·07
9·02	8·57	— 0·45

Thus the isoelectric point of the histone is at p_H 8·51.

If a solution of histone sulphate is adjusted to this p_H by the addition of sodium hydroxide, an intense cloudiness is observed and on addition of an equal volume of 85 per cent. alcohol the histone separates rapidly in granular form and can be filtered off on the pump without difficulty.

(f) *The Preformed Free Acidic and Basic Groups of the Histone Molecule and their Changes on Hydrolysis.*

The NH_2 and COOH groups must be regarded as the chief groups giving rise to ions. But the imino-group of the iminazole in histidine and the OH group in tyrosine must also be taken into account. The possibility that other groups act in this way is not excluded.

So far only some of the methods which were used to distinguish the preformed groups giving rise to ions in the protamines (p. 55) have been applied to the histones. Such are the elimination of the amino-groups by alcohol in Willstätter and W.-Leitz's method, by Sörensen's formol titration or van Slyke's method, and the estimation of the methyls taken up by the amino-groups (Edlbacher's N-methyl numbers). In the application of these methods the abnormal behaviour of the amino-group of guanidine, which is given in more detail below (p. 78), is very important.

Felix and Hartenеck (36, 37) have determined the acid and alkali combining equivalents by the electrometric method.

For estimation of the base equivalent a histone solution of known strength was treated with a known amount of sulphuric acid and the hydrogen ion concentration determined. The concentration of pure sulphuric acid of the same p_H was also determined. The difference of the concentrations of sulphuric acid in the two solutions gave the amount of sulphuric acid combined with the histone. The experiment was repeated with varying amounts of sulphuric acid. The value for the combined sulphuric acid was approximately constant within the range of certain p_H values and from it the combining equivalent for acids of the histone could be calculated. This value, under the conditions selected by Felix and Hartenеck, was 0·54 millimols sulphuric acid and 8·3—recently 9·3—equivalents for every 100 atoms of nitrogen in the histone.

By a similar titration with sodium hydroxide these authors found 11·5 (12·7) free alkali-combining equivalents to 100 atoms of nitrogen. By titration of free histone in 90 per cent. alcohol using thymolphthalein and taking as end point the first appearance of the blue colour only 8·75 groups reacted out of the 11·5 (12·7) acidic groups which had been found by electrometric titration. The difference of 2·75 might be explained by supposing that some of the acidic groups are neutralised by basic groups of the histone, probably guanidine groups, of which the dissociation is not prevented by alcohol. If the titration using thymolphthalein was performed in aqueous solution, it was found that only 4 equivalents combined. The difference is due to the different degree of dissociation in alcohol.

The amidine group of the guanidine nucleus retains its alkaline nature in alcoholic solution. Nevertheless, the carboxyl groups (especially the α-groups) can be titrated in alcoholic solution if the arginine solution is previously neutralised to bromthymol blue or azolitmin with 0·2 N hydrochloric acid. Then alcohol titration gives the same values as formaldehyde titration since in both cases an acid equivalent is titrated which is free owing to the elimination of the α-amino group (Waldschmidt-Leitz, Schäffner, and Grassmann, 193; Felix and Hartenеck, 36).

A formol titration was first done on the histone by Edlbacher when estimating the N-methyl number (i.e. number of methyl groups taken up by each 100 atoms of nitrogen). Edlbacher's (26) values have already been given on page 60. It will be seen from this table that the values are high compared with those for the non-basic proteins.

Felix and Harteneck (37) recently found 11 per cent. formol nitrogen in the thymus histone.

In the histones, as in the protamines, an amino-group of the guanidine residue can be nitrated. On acid hydrolysis of nitrohistone, nitroarginine is obtained (Kossel and Weiss, 99).

(g) *Action of Pepsin on the Histone.*

The histone is attacked by proteolytic enzymes, not only by trypsin but also, in contrast to the protamines, by pepsin which has the technical advantage that it acts under conditions under which the action of other enzymes, which may be mixed with it, is entirely or almost entirely stopped. Kossel (89) found a peptone-like substance, "*histopeptone*," in the peptic digest. This can be separated from the main bulk of the digestion products by precipitation with sodium picrate at faintly alkaline reaction, and thence isolated by the silver-baryta precipitation (p. 5). The histopeptone is obtained as sulphate. This salt contains 14·09 per cent. sulphuric acid and 17·16 per cent. nitrogen, while the sulphate-free base contains 19·98 per cent. nitrogen.

The picrate is soluble in hot water and comes out on cooling in oily drops. A solution of the histopeptone sulphate gives a biuret and Millon's reaction but not Hopkins and Cole's glyoxylic acid reaction. No sulphur can be detected by boiling with alkaline lead solution. On long boiling with acids no humin formation is observed.

Kossel (88) and Felix (33) examined the basic hydrolysis products of the histopeptone by the Kossel-Kutscher method. Their results are given in the following table:—

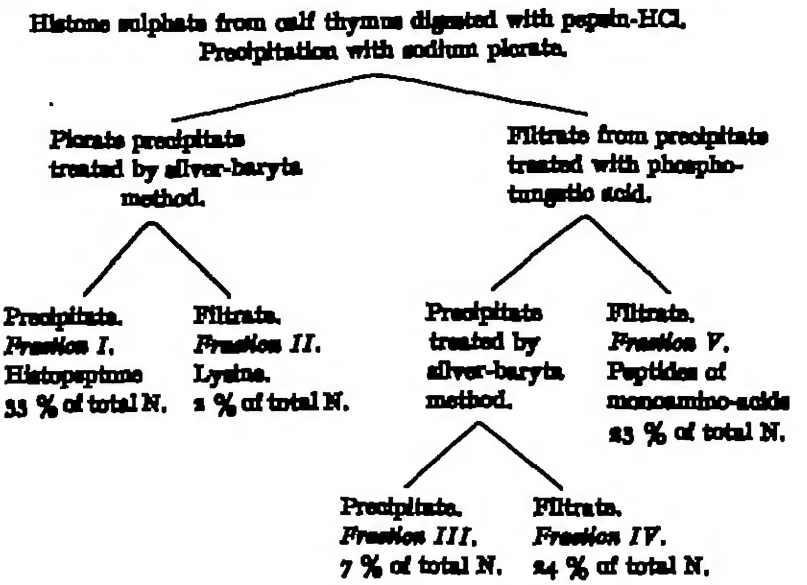
HISTOPEPTONE.

	Kossel (1898). Per Cent.	Felix (1900). Per Cent.
Total nitrogen	100	100
Ammonia nitrogen	—	—
Nitrogen absorbed on barium sulphate precipitate	—	21·4
Histidine nitrogen (maximal number)	3·7	3·6
Histidine nitrogen, weighed as picrolonate (minimal number)	4·0	2·9
Arginine nitrogen (maximal number)	27·2	28·4
Arginine nitrogen, from arginine picrolonate (minimal number)	25·8	25·9
Lysine nitrogen (maximal number)	—	15·9
Lysine nitrogen, from lysine picrate (minimal number)	17·3	12·1
Nitrogen of the monoamino-acids (difference)	27·7	27·0
Tyrosine	1·2	—

Kossel's "*Histopeptone reaction*" depends upon the formation of histopeptone. The solution to be examined for histone is digested with pepsin. The digestion mixture is rendered *very feebly* alkaline and treated with an aqueous solution of sodium picrate. A precipitate indicates the presence of the histone.

The action of pepsin can also be applied for distinguishing histone from a mixture of protamine and protein. The digestion mixture is made weakly ammoniacal and the liquid treated drop by drop with a weak ammoniacal solution of protein or Witte's peptone. If protamine is present, a precipitate is formed. A precipitate is not formed with histone.

Felix (34, 35) examined the filtrate from the picric acid precipitate. He obtained 4 fractions, two of which contained arginine-rich peptones of high molecular weight (Fractions III. and IV.) while the third (Fraction V.) contained dipeptides of monoamino-acids. Fraction II. was lysine. The lysine nitrogen amounted to 2 per cent. of the total, thus a molecule of lysine is split off from 100 atoms of nitrogen in the histone. The scheme shows the method of fractionation:—



Even if it is not accepted that these fractions are chemical individuals, their examination shows that it is probable that pepsin splits the histone into two parts, one containing arginine (Fractions I., III., IV.) and the other free from arginine. The separation of a group similar to the protamines or protones has not yet been achieved, since in none of the arginine-containing groups has the arginine content been greater than that of the histone.

The work was extended by Felix and Harteneck (37) who carried out experiments on the change in the free acidic and basic groups during peptic digestion, using the electrometric method mentioned above.

The histone preparation used for this purpose had the composition given on page 75, and the combining equivalents given on page 78. The numbers give the increase in groups per 100 nitrogen atoms:—

1. Basic groups (increase in combining power for acids)	5·2
2. Acidic groups (increase in combining power for bases)	6·0
3. Free amino-groups	2·9
4. Formol-titratable carboxyl groups	3·3
5. Alcohol-titratable carboxyl groups	3·0

Thus the action of pepsin has increased the combining power for acids and bases equally. Peptide linking is, therefore, most probable. According to Felix and Harteneck, the excess of acidic groups is too small to permit the assumption of an ester linking. On the contrary, Steudel and Ellinghaus (179) found no increase in free carboxyl groups and only increase in free amino-groups on peptic digestion of the histone sulphate.

Somewhat similar observations have been made for other proteins. Thus Waldschmidt-Leitz, Schäffner, and Grassmann (193) (p. 41) found that the action of proteolytic enzymes on clupeine produced an equal increase in the number of acidic and basic groups; the increase in amino and carboxyl groups was as 1 : 1. The same result was obtained by Waldschmidt-Leitz and Simons (194) in experiments on the peptic digestion of casein. It has already been emphasised that this result need not necessarily be explained as breakage of peptide linkages.

On the other hand, Steudel, Ellinghaus, and Gottschalk (178) found a definitely greater increase in carboxyl groups in various proteins.

CHAPTER IV.

HISTONES FROM SPERM.

Histones are found in the ripe sperm of certain classes of vertebrates and invertebrates. Substances in the *unripe* gonads of fish have also been described which are at least very similar to the histones but which have not yet been sufficiently examined to establish their relation to the histones. The histones from ripe sperm will be considered first.

Cod (80).—The histone is prepared by hydrochloric acid extraction of the sperm obtained from the testicles, after they have been extracted with alcohol and ether and dried. The histone is obtained from the hydrochloric acid extract by salting out with sodium chloride. The precipitate is filtered off and freed from salt by dialysis when the histone goes into solution again. The histone is precipitated from this solution by ammonia. Molisch's reaction is negative and the tryptophan reaction positive. The nitrogen content is 18·5 per cent. The histopeptone reaction (p. 80) is positive. The relative proportions of the hydrolysis products are given in the table on page 84.

Tadpole, Lota vulgaris.—The preparation of the histone examined by Ehrström (30) differs from the foregoing in that the mass of spermatozoa, after drying and extraction with alcohol and ether, is rubbed up with concentrated hydrochloric acid, the action of the acid being allowed to proceed for an hour at room temperature. Three to four volumes of water are then added and the precipitate, which contains the nucleic acid, filtered off. The filtrate is neutralised with sodium hydroxide and diluted when a precipitate comes down. This is dissolved in hydrochloric acid and precipitated by ammonia. The solution and precipitation is repeated several times. The histone is insoluble in water but dissolves in acids and alkalis. Such a solution can be neutralised without the separation of the histone. The ammonia precipitate is soluble in excess of ammonia and is precipitated from this solution by ammonium chloride. If the neutral salt-containing solution is boiled, a precipitate, which is insoluble in acids, separates.

Nitric acid does not precipitate the histone. This histone is distinguished from the others by these two latter reactions. It is also distinguished from the three histones mentioned in giving a positive Molisch reaction. Hopkins and Cole's tryptophan reaction is feebly positive. The nitrogen content is lower than that of the other histones. (For basic hydrolysis products, see table on p. 84.)

Centrophorus granulosus (Kossel, 88).—Only the nitrogen distribution of this histone which does not differ from that of the other histones (cf. p. 84) is known.

Echinoderms.—Very little is known about these histones but they can be included from the general distribution of the histones from sperms.

Astropecten aurantiacus (Kossel and Edlbacher, 103).—The testicles are boiled up with alcohol and the extraction completed with ether. The dry residue is extracted in a shaking machine with 1 per cent. sulphuric acid and the extract precipitated with alcohol. The precipitate is purified through the picrate and the histone is isolated as the sulphate. This is a white powder soluble in water. The solution gives the typical ammonia precipitation, biuret and Millon reaction, but the colour reactions for tryptophan and cystine and Molisch's carbohydrate test are negative. Tyrosine can be obtained in crystalline form from the monoamino-acid fraction of the hydrolysis products. The presence of histidine is doubtful. A histidine fraction is obtained which gives a diazo reaction and is free from tyrosine, but the diazo reaction is no longer given after benzoylation (cf. Inouye, 71; Kossel and Edlbacher, 102).

A peculiarity of this histone is the low arginine content (19·4 per cent. of the total nitrogen) and the high lysine content (minimum value 11·5 per cent.). Less ammonia is formed during hydrolysis than with other histones. The histone sulphate contains 15·83 per cent. nitrogen and 12·28 per cent. sulphuric acid; this corresponds to a nitrogen content of 18·05 per cent. for the free histone.

Echinus esculentus (Kossel and Staudt, 105).—The sperm is obtained from the minced testicles by shaking with water and filtering, the mass worked up in the usual way and the histone finally converted to sulphate. This substance gives a clear solution in water. The solution is precipitated by ammonia. It gives a strong Sakaguchi reaction for arginine and Millon's reaction, but no tryptophan or Molisch reaction. The lysine content is very high (see p. 84).

Histones with essentially the same properties have also been prepared from *Echinus acutus* and *Strongylocentrotus lividus*, but the

quantities were insufficient for a detailed examination. In distinction to the above echinoid histones they gave a positive Molisch reaction.

To this group belongs *Arbacine*, a substance from the testicles of *Arbacia* examined by Mathews (124). This substance differs from the histones in that the ammonia precipitation only takes place in concentrated solutions. The nitrogen content of the sulphate is 15·91 per cent. Arbacine gives a Millon and biuret reaction and forms a precipitate with proteins in ammoniacal solution.

As mentioned above, substances are found in the unripe testicles of fish whose similarity to the histones cannot be overlooked. This is especially so in the case of a substance which Bang (6) found in the testicles of the mackerel and called scombrone. Scombrone has the elementary composition—

49·86 per cent. C; 7·23 per cent. H; 19·79 per cent. N; 0·79 per cent. S.

It has a high nitrogen content. It is precipitated by ammonia and sodium hydroxide, is easily soluble in acids, is precipitated by alkaloidal reagents (and also by picric acid) in neutral or feebly alkaline solutions and thus behaves towards these reagents like a histone.

Histone.	Percentage of Nitrogen in Histone.	Hydrolysis Products, Nitrogen as per cent. of Total Nitrogen.				Colour Reactions.			C.	H.
Erythrocytes from bird blood .	18·46	—	—	—	—	+	—	—	52·31	7·09
Thymus histone	17·46 18·35	7·46	25·17 27·1	1·79 5·8	8·04	+	—	+	52·3	7·5
Histopeptone from thymus . .	19·98	—	25·9 28·4	2·9 4·0	12·1 17·3	+	—	—	—	—
Gadus Morrhua [1] .	18·3	3·3	26·9	3·3	8·5	+	—	+	—	—
Lota [1] . . .	16·47	3·3	23·44	4·12	3·69	+	+	+	—	—
Centrophorus granulosus [1] .	—	1·7	25·4	4·5	7·1	+	+	—	—	—
Astropecten aurantiacus [1 2]	18·3	0·9	19·4	3·7	11·3	+	—	—	—	—
Echinus esculentus [1] .	—	—	23·97 21·94	7·14	14·14	+	—	—	—	—

[1] From testicles.

[2] The sulphate contained 16·83 per cent. N, and 12·28 per cent. H_2SO_4.

It is partly resistant to the action of pepsin-HCl. This could be explained on the grounds that scombrone is a compound of a protein and scombrine, the protamine of the mackerel, or a mixture of protamine and histone.

It is difficult to decide whether the "*albuminose*" prepared by Miescher (127) from unripe salmon sperm is a histone. A decision can only be arrived at by an exhaustive examination of the substance.

The most important result given by this list is the evidence that histone is not one substance but a group of substances. Certain fluctuations may be due to incomplete purification or to lack of technique, but in any case there are variations in the distribution of the basic constituents, in the ammonia formation, and the tryptophan and carbohydrate contents which are due to the source of the histone concerned.

PART III.

CHEMICAL RELATION OF THE PROTAMINES AND HISTONES TO OTHER BASIC PROTEINS.

Besides the protamines and histones other substances of protein- or peptone-like nature and basic character have been found in animal tissues, which in some ways resemble the protamines and histones in properties. It is doubtful whether they should be included in these groups as the terms "histone" and "protamine" would then be quite indefinite.

Among these substances are :—

(1) The lysine-rich cyprinine at present classed as a protamine.
(2) Thymamine.
(3) The basic peptones of the intestinal mucous membrane and lymph glands.
(4) Globin.

(1) *The Lysine-rich Cyprinine.*—In clupeine, a protamine of the salmine group, the basic character is due to the free amidine group of the guanidine, in the lysine containing protamines, as mentioned on page 56, at least one amino-group of the lysine, and in the histidine containing protamines the iminazole group must be assumed free and not concerned in the peptide linking. These groups are responsible for the basic character of the whole molecule. If the free amidine group is largely replaced by the free amino-group of lysine, or the iminazole group of histidine, a change in the reactions of the protamine concerned must occur. This is the case in the sperm of certain fish, e.g. the carp. Here the proportion of the unit arginine is diminished and a correspondingly large amount of lysine is present. In some cases 40 per cent. of the total nitrogen is contained in lysine whereas the arginine nitrogen forms only 3 to 4 per cent. of the whole (Kossel and Schenck).

Hand in hand with this change in composition the property of forming precipitates with proteins, potassium ferrocyanide and sulphosalicylic acid is lost. Other general reactions of the protamines, however, are still given, e.g. biuret and Sakaguchi reactions and precipitates with picric acid, flavianic acid, and phosphotungstic acid.

(2) *Thymamine.*—The occurrence of the histone in the thymus gland led to an examination of this nucleus-rich tissue for protamines. Nelson (132) obtained from it by a complicated method a substance, thymamine, which is apparently homogeneous and which this author regards as a protamine. The platinum salt which he analysed had a composition corresponding to the formula $C_{22}H_{42}N_8O_5 . 2HCl . PtCl_4$. Thus it contains distinctly less nitrogen in proportion to carbon than any of the protamines yet analysed. It gives the biuret reaction but not Millon's, and forms an insoluble compound with nucleic acid. No details of reactions or breakdown products, which make its inclusion in the protamine group probable, have been provided. It is more reasonable to class thymamine with the following basic peptones.

(3) *Basic Peptone-like Tissue Constituents.*—Felix (32) found a substance in the thymus gland which was characterised as a protein derivative by the biuret reaction but which contained no histidine as judged from the absence of a diazo reaction. Like thymamine it was not precipitated with potassium ferrocyanide and gave no Millon reaction.

Further investigation led Felix to the conclusion that basic peptone-like substances of a similar kind formed a group widely distributed in the animal tissues. Examination of the intestinal mucous membrane and lymph glands gave two different substances.

Preparation.—They were prepared by treating the tissue with boiling alcohol and extracting with dilute hydrochloric acid. The histone was precipitated from the hydrochloric acid extract by salting out with sodium chloride and the histone-free filtrate was precipitated with phosphotungstic acid (after removing the salt). The phosphotungstates of the bases were decomposed and converted to carbonates. These were precipitated with alcohol and the product purified by several precipitations from water and alcohol.

Hydrolysis.—Examination of the hydrolysis mixture gave the following percentages of the total nitrogen :—

	Total Base Nitrogen.	Arginine Nitrogen.	Histidine Nitrogen.	Lysine Nitrogen.
Substance from intestinal mucous membrane	54	26	11	17
Substance from lymph glands .	44	14	—	27

Properties.—The carbonates of these substances react strongly alkaline. They differ from the protamines and to some extent also

from the histones in not being salted out and not being precipitated by ammonia on cautious addition of fixed alkalis. They do not give precipitates with proteins or nucleic acid (distinction from thymamine). With potassium ferrocyanide they are precipitated neither in neutral nor acetic acid solution. They are not attacked by a trypsin preparation which acts powerfully on fibrin or histones. Tyrosine is absent and nitric acid produces no precipitate. In themselves they differ in the arginine content and in the occurrence of histidine in the substance from the intestinal mucous membrane. The substance prepared from the lymph glands gives by the Kossel-Kutscher method a "histidine fraction" but like the original substance this does not give a diazo reaction.

The absence of typical protamine and histone properties might well suggest that these compounds are not real tissue-forming substances but intermediate products of metabolism. This is supported by the very small quantity in which they are found in the tissues. The resistance to trypsin suggests that the molecule is not so large and a non-colloidal nature is inferred from the fact that they cannot be salted out.

(4) *Globin*, the protein constituent of blood pigment, was classed as a histone by Schulz (157) and Bang (6). Osato (135) by precipitating with ammonia at varying p_H showed that the optimum p_H for the precipitation of globin was p_H 8·1 and, since the optimum precipitation of globin-like proteins had been shown to coincide with the isoelectric point estimated by kataphoresis, took this hydrogen ion concentration as the isoelectric point. Schulz had already classed globin as a basic protein. Haemoglobin was considered by many workers to be a salt-like combination of the carboxyl group of the haemochromogen and the amino-group of the globin (Steudel and Plieser, 177; Kuster, 112).

Abderhalden's (1) examination of the hydrolysis products showed that globin contained almost all the units found in the proteins. Of the total nitrogen the following percentages have been calculated for the bases from Abderhalden's analyses:—

Arginine	10·29
Histidine	17·05
Lysine	4·90
Total base N	32·24

Histidine is present in greater amount than the other bases.

According to the analyses by Schulz globin contains 54·97 per cent. C; 7·20 per cent. H; 16·89 per cent. N; and 0·42 per cent. S.

At first sight the properties of globin appear similar to those of the histones. Under certain conditions globin gives a precipitate with ammonia. Like histones it is precipitated by nitric acid and potassium ferrocyanide, but not by metaphosphoric acid. According to Bang it is only incompletely precipitated by the alkaloidal reagents at neutral reaction.

But its behaviour on peptic digestion is totally different to that of the histones, for no histopeptone is formed. This suggests a fundamental difference in structure. Another distinction from the thymus histone is that it is not poisonous (see p. 95). Schulz was able to inject 2 grams globin into the jugular vein of a rabbit without its dying in the next few days. Globin does not check blood coagulation like the thymus histone.

Investigations on the basic proteins and protein derivatives suggest that the basic properties of these substances might be due to three different groups present in the free and reactive state: the amidine group of arginine, the iminazole group of histidine and the α- and ε-amino-groups of mono- and dibasic amino-acids, especially lysine. All three may work at the same time as in sturine. If one of these groups is present alone or in predominating amount, it determines primarily the properties of the basic protein concerned.

In the substances which we have classed as protamines or histones, the amidine group of arginine is the predominant, and in the monoprotamines the only active one. It is this group which imprints on the protamines and histones their basic character. But in globin the iminazole group preponderates, and in certain basic proteins of carp sperm and also in basic peptones of gland tissues other amino-groups predominate, especially those contained in lysine.

PART IV.

THE BIOLOGICAL SIGNIFICANCE OF PROTAMINES AND HISTONES.

CHEMICAL processes in living material can be studied in two ways. Either one can study the *rapidly* disappearing intermediate products which are the immediate result of food absorption, e.g. the energy exchange in muscle or the oxidation processes and similar phenomena, or one can study the *slow* processes such as tissue building and growth. The work of chemical physiologists is usually more concerned with the former, which are processes more accessible to experiment, than with the latter, whose investigation is in most cases restricted to descriptive treatment. Our knowledge of the protamines and histones has not yet attained any significance in "experimental" biochemistry. It has been gained by the second method, on a purely descriptive basis.

It applies more particularly to the processes of growth and the building up of the tissues. The protamines and histones are constituents of one of the chief organs of the cell, the nucleus, and this organ is closely connected with the processes of cell division, fertilisation and inheritance. Chemical examination of the cell nucleus reveals three different states of its substance.

(1) The first state, which appears to be the original, is a combination, as yet little investigated, of proteins with an organic group which contains purine derivatives and phosphoric acid. The structure is such that so far no one has succeeded in separating the individual parts of the complex molecule from one another without considerable decomposition. This substance is known as nuclein and is widely distributed in the animal and plant kingdoms, occurring for example in the sperm of mammals.

(2) In the course of development of animal cells a change takes place in this system which results in the formation of two poles, as it were, and the whole taking the character of a salt ("dissociation of the nucleus"). By this change the nuclear substance is made more accessible to chemical examination. We can now separate an acid

and a base without destroying the structure. The acid is nucleic acid and the other constituent is the protein converted into a base. In most cases the basic protein retains its complex structure composed of 18 to 20 units, i.e. it is converted into a histone. This can be readily seen in many tissues of vertebrates and invertebrates, e.g. in the nuclei of many glandular organs, in the red corpuscles of bird blood, or in the spermatozoa of many fish (Gadidæ) and the echinoderms.

(3) In the testes of most fish the change goes still further. During the course of spermatogenesis a large part of the monoamino-acids and in some cases even a part of the bases is split off from the protein molecule and a residue is left in which the monoamino-acid part of the protein molecule has been reduced to a small amount while the basic part predominates. The substances thus formed are the protamines.

This chemical change of the cell nucleus has so far only been observed in the animal organism. Its biological significance is unknown. From the investigations of Miescher (129) and Weiss (196) on the Rhine salmon it must be assumed that the protamine is formed by the decomposition of a higher protein. The Rhine salmon is especially suitable for the investigation of such changes in the body since the following conditions are fulfilled. The animal comes up from the sea into the river after a period of good nutrition. During its existence in fresh water supplies are completely cut off since the animal takes no food. The duration of its stay in the Rhine is from five to fifteen months. During this period the testicles in fish weighing 3500 to 10,500 grams increase from 0·105 to about 6 per cent. of the body weight. The animals form the testicles at the cost of their body substance, so that besides a disappearance of fat and glycogen a considerable decrease in the muscle tissue is observed, and since the animal must live several months by liquidation of the tissues there is a considerable daily loss in weight and a considerable loss of flesh. The protein content of the body muscle, according to Miescher, drops from 17·9–19 per cent. in March to 13·0–14·3 per cent. after the milting in January. The muscle protein is thus used for two purposes, for the daily diminishing metabolism and for the formation of the testicles. But for the latter purpose a transference of the muscle protein to the growing sexual organs is not sufficient since the new protein of the spermatozoa is found to be different from the protein of the muscle. In the sperm protein, salmine, about 90 per cent. of the total nitrogen, is present as arginine. In muscle protein this amount is considerably less.

Arginine-rich salmine might be formed from arginine-poor muscle protein in two possible ways. Either the large amount of arginine required could be built up by a synthetic process or it could be taken from the muscle protein decomposed during the fasting metabolism of the salmon. Weiss (196) carried out experiments to decide whether the amount of arginine in the part of the muscle tissue decomposed was sufficient for this purpose. He estimated the amount of arginine in the muscle of salmon moving up the Rhine. He found that a salmon of 9600 grams with about 6800 grams body muscle (cf. Miescher, *loc. cit.*, p. 139), at the time of entering the Rhine, contained about 60 grams of arginine in its body muscle. At spawning time the body weight is reduced to the neighbourhood of 9 kg. If, as estimated by Miescher,[1] the weight of the ripe testes is taken as 5 per cent. of the body weight and the protamine as 6 per cent. of the ripe testes, then the ripe testes of the animal would not contain more than 23 grams arginine. Thus only 38 per cent. of the arginine in the body muscle is required for the formation of the testes.[2] According to Miescher a female salmon during its stay in the Rhine consumes 54·74 per cent. of the protein contained in the body muscle. The amount of protein decomposed is thus fully sufficient to cover the arginine requirement for the growing testes if it is assumed that the decomposition of the protein in the male salmon amounts to only two-thirds of that in the female animal.

In a species of salmon from the Pacific Ocean, the Oncorhynchus Tschawytscha (Chinook salmon), the protamine is identical with or very closely related to salmine, but the exhaustion of the muscle substance by starvation and the transference of material to the growing gonads is still more pronounced. This animal, like the Rhine salmon, after several years' good nutrition moves from the sea to the river to spawn in fresh water and takes no food after it has left the ocean. These animals which go up the Columbia River have been studied by many workers, especially C. W. Greene (49, 50, 51, 52) and K. Greene (53). After staying in brackish water without food for one to one and a half months this salmon makes a journey of 700 to 1000 miles, often swimming against rapid currents at the rate of about 7½ miles a day, to reach the spawning place. As soon as it has spawned

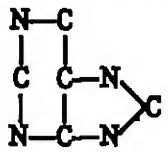
[1] In the first paper "Verhandlungen der naturforschenden Gesellschaft" in *Basel VI.*, Heft 1, 138-208 (1874), Miescher gives the weight of the testes somewhat lower, 300-400 grams "and over" for a 10-lb. salmon in November.

[2] Probably still somewhat smaller since the estimation of the protamine content of the ripe testes is rather too high.

the exhausted animal dies. This is different from the Rhine and other salmon which survive several spawning periods.

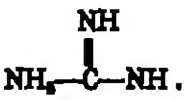
These observations support the view that the molecule of the liquidated muscle protein is divided into parts used for two physiological functions—the greater part, its *energy value*, for the enormous muscular performance and the smaller part, its *structural value*, for the formation of the gonads. This latter part is found in the sperm heads in combination with nucleic acid.

The characteristic nucleic acids of the nuclei and spermatozoa are recognised chemically by two peculiarities, the accumulation of phosphoric acid molecules and the abundance of nitrogen atoms. The nitrogen atoms are arranged alternately with carbon atoms in the form of a framework which carries mainly hydrogen atoms (adenine entirely). This is especially clear in the purine derivatives which form along with phosphoric acid the characteristic constituents of the nucleic acids in the plant and animal kingdom (Kossel).



The carbon-nitrogen skeleton of this double ring is composed of iminazole and pyrimidine rings.

In the testes of animals a similar accumulation of nitrogen atoms is also noticed in the protein residue which is attached to the nucleic acid. The histones as well as the protamines are rich in nitrogen. This characteristic is most definite in the salmine group. Out of 9 nitrogens in salmine 6 are used up in the following structure :—



Guanidine Residue of Arginine.

For insight into the character and function of the spermatozoa it is of fundamental importance to know their chemical characteristics in addition to their morphology. A peculiarity in chemical structure is seen in that part of the protoplasm which is the starting-point for the processes of reproduction and formation of new living substance.

Where this change of the proteins attains its maximum, i.e. in the protamines, the carbon linkages which are characteristic of the typical proteins are reduced to a minimum. From the whole complex protein

molecule a simple structure is formed during development of the sperm head which is composed of four or five regularly distributed units.

The chemical examination of the spermatozoa is possible since the spermatozoa of many kinds of animals can be obtained from the sperm of the testes as histologically homogeneous starting material. The ova, in which the yolk granules play an important part from the outset, do not possess this advantage. Thus, up to the present, investigations of this kind have only been possible on the spermatozoa.

ADDENDUM.

THE PHYSIOLOGICAL ACTION OF THE PROTAMINES AND HISTONES ON THE MAMMALIAN ORGANISM.

THE physiological action of the protamines and histones is also of considerable interest from the chemical point of view, since it is the result of a definite grouping of atoms which can hardly be recognised by chemical reactions. *This group is destroyed by the hydrolytic decomposition of the protamine into protone*, and is, therefore, a definite factor in the composition of the molecule. It was stated on page 45 that the protamines differ from the protones in solubilities and in forming sparingly soluble compounds with the typical proteins. A third difference is their toxic action. In this respect the histones behave like the protamines. The toxicity distinguishes—as far as is known at present—the protamines and histones from those basic proteins in which the basicity is mainly due not to a free guanidine group but to an iminazole group, e.g. globin. But the guanidine residue must not be regarded as the sole cause of the poisonous action, since the toxicity is minimal in protones and absent in arginine.

The physiological action of these substances on the dog has been examined by Thompson (185) in Kossel's laboratory. Injection of 0·1 to 0·2 gram of protamine carbonate into the circulation causes a depression of blood-pressure which can be explained as peripheral or direct influence on the vascular wall, and also a change in the respiration which must be ascribed partly to direct action on the voluntary respiratory musculature (probably simultaneously due to a central action). In addition there is a retardation of the blood coagulation and a diminution in the number of leucocytes present in the circulation. One injection of 0·2 gram clupeine carbonate into a dog weighing 10 kg. causes its death. Salmine, sturine, and histones have the same action, the fatal dose of sturine being a little larger.

The toxic action of the histones is the more remarkable since they are a constituent of the normal tissues from which they can be liberated easily even by such processes as can be assumed in pathological conditions.

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*Since I received the manuscript for translation several papers on the subject of protamines and histones have appeared. The above, which are directly connected with the matter discussed in this monograph, have therefore been added to Kossel's original bibliography.

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PRINTED IN GREAT BRITAIN BY THE UNIVERSITY PRESS, ABERDEEN